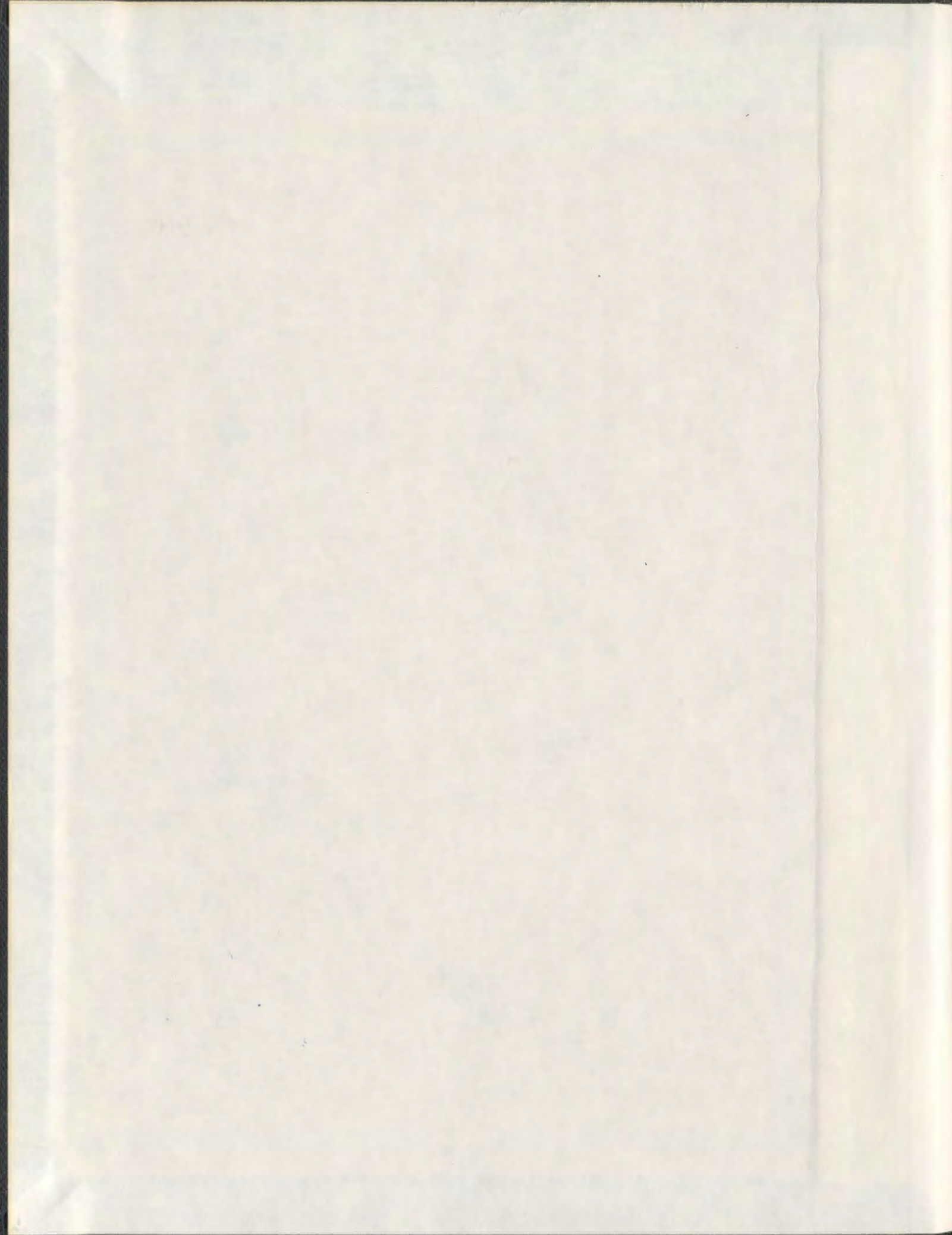


INTRAHEPATIC INNATE IMMUNITY IN HEPADNAVIRAL
INFECTION IN THE WOODCHUCK MODEL OF HEPATITIS B

CLIFFORD SCOTT GUY



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**INTRAHEPATIC INNATE IMMUNITY IN HEPADNAVIRAL
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by

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A thesis submitted to the School of Graduate Studies
in partial fulfilment of the requirements for
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Memorial University

ABSTRACT

Hepatitis B virus (HBV) causes lifelong chronic liver disease in approximately 400 million people worldwide. The causative factors underlying resolution of HBV infection and the development of chronic hepatitis B are not completely understood, although an inadequate anti-viral immune response is considered to be important in establishing chronic symptomatic infection. In this regard, previous studies have demonstrated the significance of broad strong innate and anti-HBV adaptive immune responses in inhibition of hepadnaviral replication and resolution of acute hepatitis.

The contribution of the liver to induction and maintenance of peripheral immune tolerance or to decreased immune responsiveness is increasingly recognized. These functions may in part be ascribed to the unique cellular composition of the liver, including a disproportionately high number of innate immune cell subsets and specialized antigen presenting cells. Liver cells, including hepatocytes, also appear to contribute to the immune response by interacting with naïve or activated T cells.

We hypothesized that hepatocytes can act as effector immune cells and they can directly shape hepatic immune responses and that the natural tolerizing properties of the liver may contribute to an impaired effectiveness of the anti-viral response in hepadnaviral infection. To investigate these possibilities, we utilized experimental hepadnaviral infection in woodchucks infected with woodchuck hepatitis virus (WHV), which represents the closest virological and pathogenic model of human HBV infection and hepatitis B.

In the first study, we discovered that normal hepatocytes constitutively express CD95 ligand (CD95L) and can cause death of contacted cells *via* a CD95L-CD95-dependent mechanism. Furthermore, interferon-gamma (IFN γ) and, to a lesser extent, tumour necrosis

factor-alpha (TNF α) can enhance hepatocyte CD95L-mediated cytotoxicity. This suggests that the local cytokine environment may modulate the contribution of hepatocytes to liver immunity. Subsequently, we discovered that hepatocytes also express biologically competent perforin capable of cell killing. The ability of hepatocytes to cause cell death by two different but complementary mechanisms, differentially regulated by the cytokine microenvironment, emphasized the role of hepatocytes as cytotoxic effectors while demonstrating for the first time that perforin is expressed by non-immune cells.

Further investigations revealed that hepatocyte CD95L and perforin-mediated cytotoxicity is significantly augmented during chronic hepadnaviral hepatitis and following recovery from acute infection. This could be a consequence of increased intrahepatic production of IFN γ due to virus-induced liver inflammation and strengthens the possibility that hepatocytes may actively contribute to shaping the local immune environment in hepadnaviral infection. On the other hand, a direct contribution of WHV to augmented hepatocyte cytotoxicity was excluded in a series of *in vitro* experiments applying woodchuck hepatocyte lines stably expressing WHV genome or its individual genes.

In a final study, quantitative analyses of intrahepatic hepadnaviral replication and molecular markers indicative of activation of innate and adaptive immune responses, using real-time RT-PCR assays, revealed that hepadnavirus establishes replication almost immediately following exposure to a large, liver pathogenic virus dose and that the innate response is promptly activated. However, this response appears to be insufficient in priming an effective adaptive T cell response capable of early virus elimination. These findings indicate that, in contrast to observations in HBV-infected chimpanzees, hepadnavirus is recognized immediately after invasion by the hepatic innate immune system.

Taken together, hepatocytes constitutively display cytotoxic capabilities which are susceptible to cytokine-induced augmentation. Hypothetically, this hepatic local cytotoxic activity may constrain priming of adaptive T cells by activated innate immune effectors due to deletion of virus-specific T cells. This paradigm would favour the expansion of virus replication in the liver during the prolonged incubation period typically seen in hepadnaviral infection, prior to the extrahepatic development of effective antiviral T cell responses capable of resolution of acute hepatitis. The relevance of this paradigm to the establishment of chronic hepadnaviral hepatitis will require further investigation.

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My experiences here at Memorial will never be forgotten, but as I leave to pursue other scientific endeavours, Sir Issac Newton may have said it best:

‘I do not know what I may appear to the world; but to myself I seem to have been only like a boy playing on the seashore, and diverting myself in now and then finding of a smoother pebble or a prettier shell than ordinary, whilst the great ocean of truth lay all undiscovered before me’

TABLE OF CONTENTS

	<u>Page</u>
ABSTRACT.....	i
ACKNOWLEDGEMENTS.....	iv
LIST OF TABLES.....	xi
LIST OF FIGURES.....	xii
ABBREVIATIONS.....	xiv
THESIS COAUTHORSHIP STATEMENT.....	xvii
 CHAPTER ONE:	
INTRODUCTION.....	1
1.1.1 Causative agents of viral hepatitis.....	2
1.1.2 Epidemiology of HBV infection.....	4
1.1.3 Clinical and serological profiles of HBV infection.....	5
1.1.4 Liver morphological changes in HBV infection.....	7
1.1.5 Extrahepatic complications of HBV infection.....	9
1.2 Hepatitis B Virus.....	10
1.2.1 Molecular organization.....	10
1.2.2 Ultrastructure.....	15
1.2.3 Replication strategy.....	15
1.3 Hepadnaviral family.....	16
1.3.1 Duck hepatitis B virus.....	16
1.3.2 Ground squirrel hepatitis virus.....	18
1.3.3 Woodchuck model of hepatitis B.....	18
1.3.3.1 Woodchuck hepatitis virus.....	18
1.3.3.2 Characteristics of WHV infection.....	19
1.3.3.2.1 Features of WHV-induced liver disease.....	19
1.3.3.2.2 WHV-induced hepatitis.....	21
1.3.3.2.3 Occult WHV infection.....	24
1.4 Antiviral immunity.....	25
1.4.1 Innate immunity.....	26
1.4.1.1 Antigen capture and presentation.....	27
1.4.1.2 Soluble components.....	29
1.4.1.2.1 Complement.....	29
1.4.1.2.2 Nitric oxide.....	30

1.4.1.2.3 Immunomodulatory components.....	31
1.4.1.2.3a Chemokines.....	31
1.4.1.2.3b Interferons.....	32
1.4.1.2.3c Tumour necrosis factor alpha.....	34
1.4.1.3 Cellular components.....	35
1.4.1.3.1 Neutrophils.....	35
1.4.1.3.2 Natural killer cells.....	36
1.4.1.3.3 Natural killer T cells.....	38
1.4.2 Adaptive immunity.....	40
1.4.2.1 Humoral immune responses.....	40
1.4.2.2 Cellular responses.....	41
1.5 The liver as an immune competent organ.....	43
1.5.1 Hepatic microenvironment.....	44
1.5.2 Components of the intrahepatic immune system.....	45
1.5.2.1 Professional antigen presenting cells.....	45
1.5.2.2 NK and NKT cells.....	45
1.5.2.3 Conventional T cell subsets.....	46
1.5.2.4 Non-conventional T cell subsets.....	47
1.5.2.5 Parenchymal and endothelial cells.....	47
1.5.3 Immunological function of the liver.....	49
1.5.3.1 Anti-microbial effector function.....	49
1.5.3.2 Lymphocyte retention and removal.....	50
1.5.3.3 Induction of tolerance.....	52
1.5.3.4 Induction of primary T cell immune responses.....	53
1.6 Immunopathogenesis of hepadnaviral infection.....	54
1.6.1 Innate immune response.....	54
1.6.2 Humoral immunity.....	55
1.6.3 Cell-mediated immune response.....	56
1.7 Purpose of the study.....	59
 CHAPTER TWO:	
HEPATOCYTES AS CYTOTOXIC EFFECTOR CELLS CAN INDUCE CELL DEATH BY CD95 LIGAND-MEDIATED PATHWAY.....	62
2.0 Summary.....	62
2.1 Introduction.....	63
2.2 Materials and methods.....	66
2.2.1 Cell lines.....	66
2.2.2 Preparation of hepatocytes and lymphoid cells.....	66

2.2.3	Treatment of hepatocytes with IFN γ or TNF α	67
2.2.4	RNA extraction and reverse transcription.....	67
2.2.5	Cloning of woodchuck gene sequences.....	68
2.2.6	RT-PCR and Southern blot hybridization.....	68
2.2.7	Real time RT-PCR.....	69
2.2.8	CD95L detection by Western blotting.....	69
2.2.9	Preparation of target cells for cytotoxicity assays.....	70
2.2.10	JAM cytotoxicity (DNA fragmentation) assay.....	70
2.2.11	Statistical analyses.....	71
2.3	Results.....	72
2.3.1	Hepatocytes constitutively transcribe CD95L.....	72
2.3.2	Hepatocytes display CD95L protein.....	75
2.3.3	Hepatocyte CD95L-mediated cytotoxicity.....	75
2.3.4	IFN γ and TNF α upregulate CD95L transcription in hepatocytes.....	78
2.3.5	IFN γ but not TNF α enhances hepatocyte CD95L-mediated cytotoxicity.....	79
2.3.6	Transfection with IFN γ or TNF α cDNA enhances hepatocyte CD95L-mediated cytotoxicity.....	83
2.4	Discussion.....	86
CHAPTER THREE:		
HEPATOCYTES CAN INDUCE DEATH OF CONTACTED CELLS VIA PERFORIN-DEPENDENT MECHANISM.....		91
3.0	Summary.....	91
3.1	Introduction.....	91
3.2	Materials and methods.....	94
3.2.1	Cell lines.....	94
3.2.2	Primary hepatocytes and lymphoid cells.....	94
3.2.3	Hepatocyte treatments with recombinant IFN γ or TNF α	95
3.2.4	RNA extraction and reverse transcription.....	95
3.2.5	Cloning of woodchuck gene sequences.....	95
3.2.6	RT-PCR and Southern blot analyses.....	96
3.2.7	Real time RT-PCR.....	96
3.2.8	Perforin detection by Western blotting.....	96
3.2.9	Perforin detection by flow cytometry.....	97
3.2.10	JAM cytotoxicity (DNA fragmentation) assay.....	97
3.2.11	Silencing of perforin gene by RNA interference.....	98
3.2.12	Statistical analyses.....	99
3.3	Results.....	99

3.3.1 Granule exocytosis contributes to hepatocyte killing of CD95-positive cells.....	99
3.3.2 Hepatocytes constitutively transcribe perforin and granzyme B.....	102
3.3.3 Hepatocytes are endowed in perforin protein.....	105
3.3.4 Hepatocytes kill CD95-deficient cells.....	105
3.3.5 Hepatocyte cytotoxicity is inhibited by anti-perforin antibody.....	108
3.3.6 Perforin-specific shRNA inhibits hepatocyte cytotoxicity.....	108
3.3.7 Primary hepatocytes kill CD95-deficient cells.....	112
3.3.8 Hepatocyte killing of CD95-deficient cells is not modulated by IFN γ	118
3.4 Discussion.....	118
CHAPTER FOUR:	
HEPADNAVIRAL INFECTION AUGMENTS HEPATOCYTE CYTOTOXICITY MEDIATED BY BOTH CD95-LIGAND AND PERFORIN-DEPENDENT PATHWAYS.....	125
4.0 Summary.....	125
4.1 Introduction.....	126
4.2 Materials and methods.....	129
4.2.1 Cell lines.....	129
4.2.2 WHV infection.....	129
4.2.3 Primary hepatocytes.....	130
4.2.4 Hepatocytes transfection with WHV DNA.....	132
4.2.5 Hepatocyte treatment with IFN γ	132
4.2.6 RNA extraction and reverse transcription.....	133
4.2.7 Real time RT-PCR.....	133
4.2.8 Preparation of target cells.....	133
4.2.9 JAM cytotoxicity (DNA fragmentation) assay.....	134
4.2.10 Inhibition of hepatocyte-mediated cytotoxicity.....	134
4.2.11 Statistical analyses.....	135
4.3 Results.....	135
4.3.1 Hepatocyte cytotoxic phenotype in WHV infection.....	135
4.3.2 Resolution of hepatitis is followed by persistently augmented hepatocyte cytotoxicity.....	137
4.3.3 Comparable hepatocyte cytotoxic activity accompanies CH and convalescent period after SLAH.....	139
4.3.4 Transfection with WHV X gene but not with complete WHV DNA modifies hepatocyte cytotoxic activity.....	142
4.3.5 IFN γ augments CD95L-dependent cell killing by hepatocytes transcribing complete WHV DNA but not WHV X gene alone.....	147

4.4 Discussion.....	150
CHAPTER FIVE:	
INTRAHEPATIC INNATE AND ADAPTIVE IMMUNE ACTIVITY IMMEDIATELY AFTER VIRUS INVASION AND IN THE PRE-ACUTE AND ACUTE PHASES OF HEPADNAVIRAL INFECTION.....	156
5.0 Summary.....	156
5.1 Introduction.....	157
5.2 Materials and methods.....	160
5.2.1 Study design.....	160
5.2.2 Serological assays and assessment of liver inflammation.....	160
5.2.3 Nucleic acid isolations.....	164
5.2.4 Detection of WHV DNA and RNA by real time PCR.....	164
5.2.5 Detection of WHV cccDNA.....	165
5.2.6 Identification of woodchuck cellular gene sequences.....	165
5.2.7 Analysis of woodchuck gene expression by real time PCR.....	166
5.2.8 Inactivation of WHV inoculum.....	166
5.2.9 Accession numbers of woodchuck gene sequences identified.....	168
5.3 Results.....	168
5.3.1 Serological and hepatic profiles of WHV infection.....	168
5.3.2 WHV invasion promptly activates antigen presenting cells in the liver...	173
5.3.3 Activation of hepatic NK and NKT cells immediately follows infection with WHV.....	176
5.3.4 Intrahepatic CD4+ and CD8+ T cells are quiescent for weeks after WHV infection.....	181
5.3.5 Upregulated transcription of CD95L and perforin correlates with early immune response to WHV.....	184
5.3.6 WHV invasion reproducibly induces liver immune activation at 72 hours post-exposure.....	185
5.3.7 Infectious WHV is required to activate hepatic immune response.....	189
5.4 Discussion.....	189
CHAPTER SIX:	
DISCUSSION AND FUTURE DIRECTIONS.....	200
6.1 Future directions.....	217
CHAPTER SEVEN:	
SUMMARY AND CONCLUSIONS.....	220

REFERENCES CITED.....	225
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APPENDIX A:

Woodchuck cellular gene sequences identified throughout the course of this study.....	257
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LIST OF TABLES

	<u>Page</u>
Table 4.1. Serological and histological characteristics of WHV infection in animals with self-limited acute hepatitis or chronic hepatitis investigated in this study.....	131
Table 5.1. Experimental groups according to the time elapsing between inoculation with WHV and acquisition of liver biopsy sample.....	163
Table 5.2. Primer sequences used for real-time RT-PCR quantifications of expression of woodchuck genes encoding immune cell markers, cytokines and cytotoxicity effector molecules.....	167

LIST OF FIGURES

	<u>Page</u>
Figure 1.1. Schematic diagram of the WHV genome and encoded open reading frames.....	14
Figure 2.1. Identification of CD95L mRNA and protein in hepatocytes.....	74
Figure 2.2. Hepatocyte killing of P815 and LS102.9 cell targets mediated by CD95L.....	77
Figure 2.3. Evaluation of the effects of IFN γ and TNF α on class I MHC antigen display and CD95L gene expression in hepatocytes.....	80
Figure 2.4. Exposure to IFN γ enhances hepatocyte CD95L-mediated cell killing.....	82
Figure 2.5. Transfection with IFN γ or TNF α cDNA upregulates hepatocyte CD95L-mediated cytotoxicity.....	85
Figure 3.1. Dependence of hepatocyte killing of CD95-bearing target cells on CD95L and microtubule polymerization.....	101
Figure 3.2. Identification of perforin and granzyme B in hepatocytes.....	104
Figure 3.3. Hepatocyte killing of CD95-deficient K562 target cells.....	107
Figure 3.4. Inhibition of hepatocyte killing of CD95-deficient, perforin-sensitive K562 cells by anti-perforin antibodies.....	110
Figure 3.5. Inhibition of hepatocyte-mediated killing of CD95-deficient and CD95-bearing target cells by perforin-specific shRNA.....	112
Figure 3.6. Killing of CD95-deficient target cells by primary mouse hepatocytes.....	115
Figure 3.7. Killing of CD95-deficient cells by primary woodchuck and human hepatocytes.....	117
Figure 3.8. IFN γ differentially modulates perforin gene expression in hepatocytes and PBMC.....	120
Figure 4.1. Expression of selected cellular genes known to be associated with progressing liver inflammation and those encoding molecules affiliated with hepatocyte cytotoxic activity.....	137

Figure 4.2. The resolution of acute WHV hepatitis is accompanied by increased hepatocyte cytotoxic activity.....	141
Figure 4.3. Expression levels of the cytotoxicity-affiliated genes and the cell killing potency displayed by hepatocytes after resolution of acute WHV infection and during chronic WHV hepatitis.....	144
Figure 4.4. Expression of the cytotoxicity-affiliated genes and the cell killing capacity of woodchuck hepatocytes transfected with complete WHV genome or WHV subgenomic sequences encoding individual viral proteins.....	146
Figure 4.5. IFN γ augments CD95L-dependent cell killing mediated by hepatocytes expressing complete WHV genome but not by those transfected with the WHV X gene.....	149
Figure 5.1. Schematic representation of the time-line of acquisition of liver tissue samples from WHV-infected woodchucks	162
Figure 5.2. Serological and molecular analyses of WHV infection markers following inoculation with 1.1×10^{10} WHV vge per dose.....	170
Figure 5.3. WHV infection upregulates intrahepatic genes indicative of activation of antigen presenting cells.....	175
Figure 5.4. Expression profiles of hepatic genes indicative of activation of NK and NKT cell subsets in woodchucks inoculated with WHV.....	179
Figure 5.5. Expression of T cell-affiliated genes in sequential liver samples collected from animals infected with a liver pathogenic dose of WHV.....	183
Figure 5.6. Challenge with WHV, but not with inactivated hepadnavirus upregulates genes indicative of intrahepatic immune response activation at 72 hours post-infection.....	188
Figure 6.1. GFP-CD95L fusion protein exhibits cytoplasmic rather than membrane localization in WCM-260 hepatocytes.....	203
Figure 6.2. Neuraminidase treatment augments hepatocyte-mediated cytotoxicity against CD95-positive and CD95-negative target cells.....	207
Figure 6.3. Hepadnavirus augments hepatocyte-mediated cytotoxicity 72 hours post-infection.....	213

ABBREVIATIONS

α -galcer	α -galactosylceramide
ADCC	antibody-dependent cellular cytotoxicity
AH	acute hepatitis
AICD	activation-induced cell death
ALT	alanine aminotransaminase
anti-HBc	antibodies to hepatitis B virus core antigen
anti-HBe	antibodies to hepatitis B virus e antigen
anti-HBs	antibodies to hepatitis B virus surface antigen
anti-WHc	antibodies to woodchuck hepatitis virus core antigen
anti-WHs	antibodies to woodchuck hepatitis virus surface antigen
APC	antigen presenting cell
ASGPR	asialoglycoprotein receptor
ASHV	arctic squirrel hepatitis virus
bp	base pairs
C	core gene of HBV or WHV
cccDNA	covalently closed circular DNA
CD40L	CD40 ligand
CDC	complement-dependent cytotoxicity
CH	chronic hepatitis
CMV	cytomegalovirus
cpm	counts per minute
CREB	cyclic AMP response element binding protein
CRP	complement reactive protein
CTL	cytotoxic T lymphocyte
DC	dendritic cells
DHBV	duck hepatitis B virus
ECL	enhanced chemiluminescence
ER	endoplasmic reticulum
GFP	green fluorescent protein
GSHV	ground squirrel hepatitis virus
HAV	hepatitis A virus
HBcAg	hepatitis B virus core antigen
HBeAg	hepatitis B virus e antigen
HBsAg	hepatitis B virus surface antigen
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HDV	hepatitis D virus
HEV	hepatitis E virus
HGV	hepatitis G virus
HIV	human immunodeficiency virus
HRP	horseradish peroxidase
HSV-2	herpes simplex virus type-2
ICAM-1	intercellular adhesion molecule 1

IFN α	interferon alpha
IFN β	interferon beta
IFN γ	interferon gamma
Ig	immunoglobulin
IHL	intrahepatic lymphocyte
IL-2	interleukin-2
IL-4	interleukin-4
IL-6	interleukin-6
IL-8	interleukin-8
IL-10	interleukin-10
IL-12	interleukin-12
IL-12R	interleukin-12 receptor
IL-15	interleukin-15
IL-18	interleukin-18
i.v.	intravenous
kb	kilobase pairs
KIR	killer Ig-like
LBx	liver biopsy
LCMV	lymphocytic choriomeningitis virus
LFA-1	lymphocyte function antigen 1
LPS	lipopolysaccharide
LSEC	liver sinusoidal endothelial cells
mAb	monoclonal antibody
MBL	mannan binding lectin
MHC	major histocompatibility complex
NAH	nucleic acid hybridization
NF κ B	nuclear factor kappa B
NK	natural killer cell
NKT	natural killer T cell
nm	nanometres
NO	nitric oxide
NOS	nitric oxide synthase
OAS	2',5'-oligo adenylate synthase
ORF	open reading frame
P	polymerase gene of HBV or WHV
P-WHs	plasmid WHV envelope gene
P-WHc	plasmid WHV nucleocapsid protein
P-WHX	plasmid WHV X gene
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
pgRNA	pre-genomic RNA
p.i.	post-infection
PKR	double-stranded RNA dependent protein kinase
preS1	large surface protein
preS2	middle surface protein
PRR	pathogen recognition receptor

rcDNA	relaxed circular DNA
RNAi	ribonucleic acid interference
RT	reverse transcriptase
RT-PCR	reverse transcriptase-polymerase chain reaction
rwIFN γ	recombinant woodchuck interferon gamma
rwTNF α	recombinant woodchuck tumour necrosis factor alpha
S	surface/envelope gene of HBV or WHV
SDH	sorbitol dehydrogenase
SEM	standard error mean
shRNA	short hairpin ribonucleic acid
SLAH	self-limited acute hepatitis
TCR	T-cell receptor
TGF β	transforming growth factor beta
T _H	helper T cell
T _{H1}	T helper type 1
T _{H2}	T helper type 2
TLR	toll-like receptor
TNF α	tumour necrosis factor alpha
TNFR	tumour necrosis factor receptor
TRAIL	TNF-related apoptosis inducing ligand
TRAIL-R	TNF-related apoptosis inducing ligand receptor
U	units
UV	ultraviolet
vge	virus genome equivalents
WHcAg	woodchuck hepatitis virus core antigen
WHeAg	woodchuck hepatitis virus e antigen
WHsAg	woodchuck hepatitis virus surface antigen
WHV	woodchuck hepatitis virus
wIFN γ	woodchuck interferon gamma
wTNF α	woodchuck tumour necrosis factor alpha
X	X gene of HBV or WHV

THESIS CO-AUTHORSHIP STATEMENT

This thesis is comprised of 7 chapters. Chapter 1 introduces the background and rationale for the thesis work. Chapters 2-5 contain the original data compiled from each of the projects completed for the thesis. Chapter 6 gives a discussion which highlights the cohesiveness of the projects completed during this study, while Chapter 7 summarizes the major conclusions of the work.

A great majority of the work described in this thesis was performed by the author, whose primary role was a contribution to the design of the research methodology, development of analytical methods and determination of woodchuck gene sequences, data collection and analysis, and manuscript preparation. Chapter 2 was published as a first-authored paper entitled "**Hepatocytes as cytotoxic effector cells can induce cell death by CD95 ligand-mediated pathway**" in *Hepatology*, 2006, volume 43(6), pp. 1231-1240. This paper was accompanied by an Editor's commentary entitled "**Killer hepatocytes fight back**" in *Hepatology*, 2006, volume 44(2), p285. The work comprising Chapter 3 has been accepted for publication as a first-authored manuscript in *Hepatology*, 2008 (in press). The author appreciates and recognizes those contributions made by others as follows: stably transfected woodchuck hepatocyte cell lines utilized throughout the current project were generated by Jinguo Wang; histological assessment of liver tissues was performed by Dr. Thomas Michalak; surgical assistance during acquisition of liver biopsy samples was provided by Colleen Trelegan; Western blot analyses presented in Chapter 3 were performed by Sherri Rankin; serological assays presented in Chapter 5 were conducted by Norma Churchill; determination of cccDNA as outlined in Chapter 5 was performed by Patricia Mulrooney-Cousins.

CHAPTER ONE: INTRODUCTION

Hepatitis B virus (HBV) is the largest causative agent of virally-induced liver disease worldwide and is responsible for significant morbidity and mortality. Although the existence of parenterally transferred hepatitis had been realized as early as the 1940s (Havens, 1944; MacCallum and Bradley, 1944), identification of HBV subviral particles constituted by envelope lipo-proteins did not occur until the 1960s with the discovery of Australia antigen (Blumberg et al., 1967). The virion ultrastructure was formally demonstrated in 1970 by Dane (Dane et al., 1970). This was further followed by immunological analysis of viral proteins (Milich and McLachlan, 1986; Milich et al., 1986) and molecular analysis of the virus genome (Chisari et al., 1989). Highly restricted host-specificity of the virus led to the search for appropriate animal models, which succeeded in the identification of natural models (Summers et al., 1978), and invaluable transgenic mouse systems of hepatitis B (reviewed in Chisari and Ferrari, 1995). Subsequent investigations using these models have lead to our current understanding that HBV is not a cytopathic virus, rather it is the host's immune responses directed against viral antigens which cause the development of hepatitis (reviewed in Guidotti and Chisari, 2001). Furthermore, it was established that host genetic factors, the dose of invading virus, age at the time of infection, and interactions between HBV and the host's immune system strongly influence the outcome of hepadnavirus hepatitis, leading either to recovery from symptomatic infection or progression to chronic hepatitis (CH) (reviewed in Webster and Bertoletti, 2002).

1.1.1 Causative agents of viral hepatitis

Liver inflammation characterized by varied degrees of hepatocyte damage, lymphomonocytic infiltrations and coexisting liver regeneration can be caused by one of six presently recognized, albeit unrelated viruses. They are designated as A, B, C, D, E and G, and they belong to several different viral families. Their classification is predominantly determined on the basis of the type of nucleic acid constituting their genomes, and primary routes of transmission.

Hepatitis A virus (HAV) and hepatitis E virus (HEV) are both enteric RNA viruses which are disseminated via the faecal-oral route. They are associated with unsanitary conditions, including virus-contaminated food and water supplies. Although enteric hepatitis has been recognized for centuries, its viral etiology was only established in 1973 (Feinstone et al., 1973). Similarly, HEV was not formally recognized until 1990 (Reyes et al., 1990). HAV and HEV infections are usually self-limited and result in complete recovery. However, HEV induces a surprisingly high mortality rate among pregnant women with severe or fulminant hepatitis observed in 10-20% of the cases (reviewed in (Koziel, 1996)). The single largest causative agent of viral hepatitis is HBV, a DNA virus, with an estimated 400 million serum HBsAg-positive carriers worldwide. Hepatitis C virus (HCV), comprised of a positively stranded RNA genome, affects a further 170 million. Both pathogens are spread mainly through body fluids and vertically from mother to infant. These viruses mediate significant rates of morbidity and mortality worldwide due to their inherent capacity to establish chronic infections. Approximately 10% of patients infected with HBV and 50-85% of those infected with HCV develop CH with subsequent development of

cirrhosis and hepatocellular carcinoma (HCC). While prophylactic vaccines are available for prevention of HBV, such strategies have not yet been developed against HCV infection. Thus, chronic HBV and HCV infections represent substantial healthcare and sociological burdens requiring antiviral treatment of the affected patients.

Hepatitis D virus (HDV) is an RNA virus which is unique among the hepatitis viruses since it requires HBV to establish infection (Rizzetto et al., 1980). Initially believed to represent an unidentified HBV antigen, it was further proven to be a novel virus which may replicate independently of HBV. HDV requires HBV or woodchuck hepatitis virus (WHV) envelope proteins to initiate infection, and for virion assembly. Patients who are co-infected with HBV and HDV are at an increasing risk for development of severe liver damage. In these cases, the synergistic effect of immune responses mounted against both viruses and the subsequent liver injury is greater than that observed during HBV infection alone (Acorn et al., 1995; Chu et al., 1989; Karayiannis et al., 1990). However, since both viruses share the same envelope proteins, vaccination against HBV also prevents HDV infection.

Hepatitis G virus (HGV) is an RNA virus originally identified as a causative agent of non A-E hepatitis (Linnen et al., 1996). HGV shares genomic and structural similarities with HCV (Bukh et al., 1999; Simons et al., 1996). In contrast with HCV, however, HGV causes a mild self-limiting infection which is of limited pathological importance (Linnen et al., 1996). The most recently identified TT virus (TTV) was initially thought to be a cause of non-A-G cryptogenic hepatitis (Nishizawa et al., 1997). Subsequent analysis revealed a non-enveloped negative stranded DNA genome

(Mushahwar et al., 1999). The viral proteins and replicative strategy of this virus remain largely unknown. Identifiable in primates and across a wide range of human populations, the infection is particularly prevalent in multiply transfused patients. While TTV likely has the ability to infect the liver, the relationship between TTV and hepatitis has so far been a rare phenomenon.

1.1.2 Epidemiology of HBV infection

Despite the availability of an effective prophylactic vaccine, chronic HBV infection significantly contributes to worldwide mortality. Current estimates indicate that 60% of all cases of HCC occur in patients who are chronically infected with HBV. Globally, the rates of HBV infection vary greatly in different geographical regions. In highly endemic regions, such as Southeast Asia and sub-Saharan Africa, where the majority of infections occur due to vertical transmission, 15-20% of the total population may experience chronic infection with HBV (Chen and Sung, 1978). Although prophylactic vaccination does not represent an effective strategy to reduce the rates of already established chronic infection (reviewed in (Lok et al., 1989), the advent of a mass immunization program in Taiwan beginning in 1984 has dramatically reduced the rates of chronic hepatitis B in babies born to infectious carrier mothers (Hsu et al., 1988). Due to universal vaccination in children, the developed world, including Europe and North America, have experienced declines in the rates of HBV infection among individuals aged 0-19 years (reviewed in (Fung and Lok, 2005). However, increased rates of transmission have been observed in high risk groups, including intravenous drug users and male homosexuals (reviewed in (Fung and Lok,

2005). Furthermore, the rate of infection remains endemically high in some groups including native Canadian populations and those who have recently immigrated to Canada from areas where chronic HBV infection is prevalent (Minuk and Uhanova, 2003).

1.1.3 Clinical and serological profiles of HBV infection

The course of HBV-induced liver disease is variable. The manifestation may include an inapparent disease state which progresses without clinical symptoms, but which is biochemically and serologically evident. Alternatively, patients may develop symptomatic acute hepatitis (AH) which may result in recovery, progression to CH, or rarely to the development of highly lethal fulminant hepatitis.

Clinically evident acute HBV infection is identifiable in patients following a prolonged incubation period of 45-120 days. Prodromal infection, characterized by mild fever, anorexia, nausea and vomiting, precedes the icteric phase of disease. The appearance of serum HBV surface antigen (HBsAg) becomes evident 7-9 weeks post-infection (p.i.) and may rise to 200 µg/mL or more, while HBV DNA is detectable in serum 2-3 weeks before the onset of HBsAg (Kaneko et al., 1990).

Typically HBV e antigen (HBeAg) and antibodies to WHV core antigen (anti-WHc) appear subsequent to HBsAg and concurrent with elevations in aminotransferases (reviewed in (Hoofnagle, 1981). Although seroconversion to anti-HBe antibody (anti-HBe) positivity is associated with a reduction in the level of serum HBV DNA and normalization of liver function, it has been firmly demonstrated that HBV DNA persists indefinitely in patients following recovery when sensitive polymerase chain reaction (PCR)-based assays are utilized (Michalak et al., 1994). The

clearance of HBsAg and the appearance of anti-HBs antibodies (anti-HBs) signifies the end of AH, with anti-HBs persisting for life in most patients.

Conversely, chronic hepatitis B is characterized by serum HBsAg positivity for longer than 6 months with co-incident production of anti-HBc. Typically 5-10% of adults who become infected with HBV develop chronic liver inflammation, which may occur as either persistent (mild) or active (aggressive) hepatitis. However, patients with acute icteric hepatitis tend to develop CH less frequently. An additional category of chronic HBV infection includes those patients who are persistently serum HBsAg positive, but are without apparent clinical symptoms of hepatitis. These “healthy” chronic carriers are anti-HBc positive and display minimal to mild liver tissue histological abnormalities (De Franchis et al., 1980).

A predominant factor influencing the development of CH type B is the age at which infection is initiated, with up to 90% of infected neonates displaying chronic HBV-induced liver disease (Okada et al., 1976). In areas of endemically high rates of vertical transmission, such as Southeast Asia, epidemiological studies strongly support the relationship between neonatal infection and CH, with the subsequent development of hepatocellular carcinoma (HCC) (Beasley, 1988; Beasley et al., 1981). Although the mechanisms underlying the development of HCC in HBV-positive patients are not completely understood, HBV DNA integration into the host genome, chronic liver inflammation and liver tissue regeneration have been identified as constant features (Murakami et al., 2005). Virus genome integration may disrupt tumor suppressor genes or facilitate activation of oncogenes leading to the development of HCC (Feitelson et al., 2002). In addition, HBV X protein has been demonstrated to possess

transactivating ability (Rossner, 1992) and may influence the development of malignancy via modulation of cellular machinery or rendering cells resistant to apoptotic mechanisms.

1.1.4 Liver morphological changes in HBV infection

HBV-induced liver disease is characterized by hepatocyte destruction which is mediated by inflammatory infiltrate. The histological hallmarks of AH type B are intralobular foci of necrotic or apoptotic hepatocytes accompanied by inflammatory infiltrations consisting of lymphocytes, macrophages, polymorphonuclear leukocytes and, rarely, plasma cells. Hepatocellular damage is characterized by ballooning degeneration, wherein hepatocytes exhibit expansion of the endoplasmic reticulum and become swollen with fragmenting membranes, and disintegrating nuclei. In addition, hepatocytes can undergo apoptosis (Lau et al., 1998). The induction of apoptosis may occur via multiple mechanisms, including ligation of death receptors expressed on hepatocyte cell membranes, or as a result of oxidative stress (reviewed in (Malhi et al., 2006)). In either case, cell shrinkage and acidophilic changes in the hepatocyte cytoplasm precede the penultimate occurrence of apoptotic bodies following cellular fragmentation. The largest of these, representing entire apoptotic cells or Councilman bodies, which were originally described by Councilman in yellow fever (Councilman, 1890). Interestingly, Councilman bodies may also be observed within centrilobular hepatocytes in the absence of lymphocytic infiltrations. In addition, regenerative changes are usually observed, including binucleated hepatocytes, while cholestatic obstruction is rarely seen.

Fulminant hepatitis, representing less than 1% of hepatitis induced by HBV, is characterized by large areas of confluent necrosis which is often bridging between central veins. This submassive necrosis may include extensive parenchymal collapse followed by nodular regeneration with distorted architecture and postnecrotic scarring including the appearance of collagen and elastin fibers (Thung et al., 1982).

CH type B is associated with protracted inflammatory destruction of hepatocytes progressing finally to liver fibrosis and cirrhosis. Evaluation of histological damage using Knodell scores (Ishak et al., 1995) consider both inflammatory components as well as the extent of fibrosis. Inflammatory activity is classified according to the occurrence in three lobular zones: (1) interface zone, (2) portal area, and (3) hepatocellular parenchyma. Interface hepatitis, or piecemeal necrosis, includes the destruction of hepatocytes lining the liver lobule limiting plate. Such destruction may be focal or extensive, with lymphocytic infiltrators penetrating into the hepatic parenchyma or to other portal areas. Portal infiltration is characterized by extensive aggregates of both lymphocytes with macrophages and dendritic cells, and occasionally observed formation of germinal centers. Parenchymal infiltrates are histologically similar in both AH and CH, with some differences. Although foci consisting of lymphocytes and macrophages are evident, concurrent with apoptotic bodies and "drop-out" hepatocytes (Afford et al., 1995), such foci observed during CH often contain an abundance of plasma cells not observed during acute infection. In addition, fibrosis characterizes the extent of liver injury and determines prognosis of chronic HBV infection. Expansion of the portal areas by collagen formation may surround regenerative nodules forming rosettes of hepatocytes. Continued

inflammation results in the formation of fibrous septa which extend between portal areas. This distorted liver architecture is recognized as cirrhosis. Cases of CH type B are often characterized by the presence of ground glass hepatocytes (Huang and Neurath, 1979). These slightly larger hepatocytes are sometimes seen singularly or in clusters and are recognizable by pale, eosinophilic, granular cytoplasmic inclusions which displace the nucleus. Such inclusions are strongly reactive with antibodies to HBsAg (anti-HBs) (Meuleman et al., 2006; Wang et al., 2003a; Wu and Lam, 1979). Electron microscopy examination reveals both tubular and spherical forms of HBsAg particles in the endoplasmic reticulum (ER) (Huang and Neurath, 1979; Meuleman et al., 2006).

1.1.5 Extrahepatic complications of HBV infection

Extrahepatic manifestations of HBV infection are observed in 10-20% of HBV-infected patients, and are clinically represented by: (1) transient serum sickness-like syndrome, (2) acute necrotizing vasculitis, (3) polyarthritis nodosa, and (4) glomerulonephritis.

Transient sickness is often represented by cutaneous rash. Polyarthritis may precede the development of jaundice by several weeks (Alpert et al., 1971). The condition coincides with decreased levels of complement in the serum of HBsAg-positive patients (Alpert et al., 1971) and circulating immune complexes containing HBsAg, anti-HBs and complement.

Up to 50% of patients who are diagnosed with acute necrotizing vasculitis are serum HBsAg-positive. This disease is rather uncommon in chronic carriers and is

more closely associated with recent HBV infection (Guillevin et al., 1992). Periarteritis nodosa is a disease affecting small and medium sized arteries, normally associated with chronic hepatitis B. Mortality rates of 40% are observed within three years if left untreated. Pathogenic role of HBsAg-anti-HBs immune complexes in this disease has been documented (Michalak, 1978).

HBV-associated nephropathy, i.e. membrano-proliferative or membranous glomerulonephritis, is identified in both children and adults while apparent liver disease may be slight or absent. Areas experiencing a high endemic rate of HBV infection report a higher frequency of HBV-induced glomerulonephritis, which correlates with serum HBsAg positivity (Gilbert and Wiggelinkhuizen, 1994). Deposition of immune complexes containing HBsAg, HBcAg and/or HBeAg can be detected in basement membranes of kidney glomeruli in this disease (Lai et al., 1994; Slusarczyk et al., 1980).

1.2 HEPATITIS B VIRUS

1.2.1 Molecular organization

Viruses of the hepadnaviridae family, which encompasses orthohepadnaviridae (mammalian viruses) and avihepadnaviridae (avian viruses) are among the smallest DNA viruses recognized. HBV, the prototypic member of the hepadnaviridae family, has a distinctly compact genome which is a circular 3.2 kilobase (kb)-long, partially double stranded DNA structure referred to as relaxed circular DNA (rcDNA). This unique viral genome is maintained by a short cohesive overlap between the 5'-ends of the plus and minus DNA strands. Both DNA strands bear 5' modifications which are

necessary for initiation of virus replication; the 5'-end of the DNA minus strand contains a covalently linked protein, whereas the plus strand has a 5'-RNA oligonucleotide primer attached (Lien et al., 1986; Loeb et al., 1991).

The genome contains four open reading frames (ORF) encoding the four major translation products: the virus envelope or surface (S), core (C), polymerase (P) and X proteins. Three envelope proteins are encoded by the S ORF, which contains three in-frame start codons; however these proteins are derived from only two mRNA transcripts (2.4 and 2.1 kb) (see Figure 1.1). In consequence, these proteins share a common carboxy-terminus while differing at the amino-termini, and are referred to as large (preS1), middle (preS2) and major or small (S) surface proteins (Gerlich et al., 1992).

The C ORF, contained within the 3.5 kb pregenomic mRNA transcript, encodes the virus nucleocapsid protein (HBcAg), as well as an additional protein displaying e antigen specificity (HBeAg). HBeAg is generated by post translational modifications of the core protein. Although both proteins share nearly identical amino acid sequences, except for an additional ten amino acid sequence encoded by the pre-core region and deletion of 34 residues from the carboxy terminus of the C genomic region, they stimulate the production of distinct antibodies; i.e., anti-HBe and anti-HBc antibodies (Ou et al., 1986). The core protein is localized within infected hepatocytes and virions, while HBeAg can be detectable both within hepatocytes and in circulation, but does not comprise the virion (Uy et al., 1986).

Transcription of the P ORF, also derived from the 3.5 kb pregenomic mRNA transcript, yields a polypeptide which displays viral reverse transcriptase (RT), and

RNase and DNA polymerase activities (Wei and Peterson, 1996). In addition, this protein contains a packaging signal and facilitates reverse transcription of the viral pregenome, thus it is indispensable for hepadnavirus replication (Bavand and Laub, 1988).

The smallest HBV transcript, 0.7 kb in length, encodes the X protein which appears to play multiple roles within the context of cellular activation and oncogenesis. The influence of viral X protein on host regulatory elements may include interactions with cyclic AMP (cAMP) response element binding protein (CREB) (Maguire et al., 1991) or with DNA repair enzymes (Becker et al., 1998). Furthermore, X protein has been demonstrated to activate several cytoplasmic signaling pathways including Ras/MAPK (Klein and Schneider, 1997) and JNK (Benn et al., 1996). This may be due to its inherent ability to release intracellular calcium, thus influencing multiple downstream signaling components responsible for, among other things, enhancement of viral replication (Lara-Pezzi et al., 1998a; Lee et al., 1995).

1.2.2 Ultrastructure

Analysis of sera from HBV-infected patients by electron microscopy can reveal three distinct forms of viral particles. The complete virion or Dane particle (Dane et al., 1970) occurs as a 42-47 nm sphere that has a distinct outer envelope and internal virus capsid. The viral envelope is comprised of proteins carrying HBsAg reactivity as well as host-derived lipoproteins, while the viral DNA genome and polymerase are packaged within the nucleocapsid. Although Dane particles may exist in the blood at levels of 10^9 /ml or greater, subviral particles comprised of HBV envelope proteins may occur at astonishingly high levels of 50-300 $\mu\text{g/ml}$ or 10^{12} particles/ml. These particles may exist as spherical (20 nm in diameter) or tubular (up to 230-nm long) forms which are devoid of nucleic acids and, hence, are non-infectious, but are highly immunogenic. Since antibodies directed against HBsAg provide protective immunity, preparations of the subviral HBsAg particles from the blood of infected patients were utilized as HBV vaccines before the advent of the recombinant HBsAg vaccines.

1.2.3 Replication strategy

Hepadnaviral infection is initiated by virus attachment to an as yet unidentified cellular receptor(s). Fusion of the virus envelope with the cells plasma membrane facilitates entry of the nucleocapsid into the cytoplasm with its subsequent transport to the nucleus (Hild et al., 1998). The virus rcDNA genome is repaired by host DNA polymerases and ligases (Kock and Schlicht, 1993) yielding covalently closed circular DNA (cccDNA). Since cccDNA represents the template from which viral replicative intermediates are transcribed, the detection of cccDNA provides definitive proof of

hepadnaviral replication (Mason et al., 1983). Hepadnaviral replication is unique among DNA viruses, since transcription of the cccDNA template by host RNA polymerase yields a full-length viral genome or pre-genomic RNA (pgRNA). This pgRNA transcript is packaged within the nucleocapsid together with viral polymerase, and functions as the transcriptional template from which minus-strand DNA is reverse transcribed. Although core proteins may form capsids devoid of nucleic acids, encapsidation of subgenomic RNA transcripts is excluded since only the pgRNA contains the 5'-region (denoted ϵ) necessary for encapsidation (Enders et al., 1987). Degradation of pgRNA by viral polymerase is followed by plus DNA strand synthesis, utilizing the minus strand DNA as template (Summers and Mason, 1982). In consequence, mature nucleocapsid particles containing the rcDNA genome may be recycled to the nucleus or packaged into virions. Studies using duck hepatitis B virus (DHBV) revealed that nuclear translocation of newly formed nucleocapsids represent the default pathway during the initial stages of replication, and precede the synthesis and accumulation of viral envelope proteins (Summers et al., 1990) which ultimately result in virion assembly and their secretion.

1.3 HEPADNAVIRAL FAMILY

1.3.1 Duck Hepatitis B Virus

DHBV is the prototypic member of the genus *avihepadnaviridae*, which is also comprised of snow goose hepatitis B virus (SGHBV) as well as lesser known viral species isolated from geese, storks and varied duck species (reviewed in (Jilbert and Kotlarski, 2000). DHBV is recognized, along with WHV (see Section 1.3.3), as the

most widely accepted models for the study of HBV infection. However, important differences have been identified between DHBV and the mammalian hepadnaviruses. For example, DHBV lacks an X gene, synthesizes two (not three) envelope proteins, and the surface of DHBV particles do not exist as freely circulating filaments.

The validity of DHBV as a model of HBV infection is supported by similar age and dose-related outcomes of liver disease as those observed for HBV and WHV infections. Anti-DHBV humoral responses can be detected against both core and surface proteins (Halpern et al., 1987; Jilbert et al., 1998), while viral clearance appears to proceed mainly via non-cytolytic mechanisms (Jilbert et al., 1998; Jilbert et al., 1992).

The utility of DHBV as an experimental model to explore the mechanisms of immunopathology is reduced due to several key restrictions. Currently, limited knowledge of the duck immune system and the lack of reagents to specifically identify lymphoid cell subsets and other critical components of the immune system precludes investigating their involvement in the clearance of DHBV. Furthermore, histological damage observed during chronic DHBV infection is markedly less severe than that during HBV infection (Jilbert et al., 1998), with a distinct lack of progressive cirrhosis and development of HCC. Thus, DHBV does not facilitate analysis of the pathogenic or oncocytic mechanisms underlying the development of chronic liver injury. Notwithstanding these limitations, the application of primary duck hepatocyte cultures to analyze DHBV-host cell interactions has provided important insights into a better understanding of hepadnaviral replication (Tuttleman et al., 1986), and has proven useful for evaluation of anti-viral therapies (Suzuki et al., 1988).

1.3.2 Ground Squirrel Hepatitis Virus

Ground squirrel hepatitis virus (GSHV) was originally identified in Beechey ground squirrels. GSHV virions are smaller in size than HBV, while an additional restriction enzyme cleavage site (Pvu II) is located within the GSHV dsDNA genome (Marion et al., 1980). Relatively recent identification of another hepadnavirus, which endemically infects arctic ground squirrels (ASHV) (Testut et al., 1996), indicates further divergence of the hepadnaviral family. Interestingly, both hepadnaviruses cause similar liver pathologies and both are infectious to woodchucks (Seeger et al., 1991). However, when the course of chronic liver disease induced by infection of woodchucks with either GSHV or WHV was compared, it was found that while GSHV infection resulted in similar viral loads and inflammatory disease. However, the ability of GSHV to induce HCC was noticeably lower than that for WHV (Seeger et al., 1991).

1.3.3 WOODCHUCK MODEL OF HEPATITIS B

1.3.3.1 Woodchuck Hepatitis Virus

The discovery of WHV in a colony of captive woodchucks (*Marmota monax*) at the Philadelphia Zoological Gardens (Summers et al., 1978) provided an invaluable, mammalian natural model of HBV infection and hepatitis B. This model allows for comprehensive studies regarding molecular, immunological and pathological features of hepadnaviral infection. WHV shares considerable structural and molecular homology with HBV, including genomic structure, antigen cross-reactivity, tissue tropism and pathological features of liver disease which may culminate in the development of HCC (reviewed in (Michalak, 1998).

WHV virions have a diameter of 45 nm and are slightly larger than HBV. The viral envelope is comprised of three viral glycoproteins carrying WHV surface antigen (WHsAg) specificity. The 27-nm nucleocapsid, which displays both core (WHcAg) and e (WHeAg) antigen reactivities, encapsulates the virus genome and viral polymerase, a feature conserved among the hepadnaviruses. The high degree of similarity between WHV and HBV genome sequences (overall 65%) is reflected in a high degree of antigenic cross-reactivity between their envelope and core proteins. In consequence, commercial assays for the detection of HBsAg, anti-HBs and anti-WHc can be utilized to identify respective WHV proteins or antibody specificities

1.3.3.2 CHARACTERISTICS OF WHV INFECTION

1.3.3.2.1 Features of WHV-induced liver disease

Notwithstanding minor variability, and an apparent lack of progressing liver cirrhosis, adult woodchucks infected with WHV have similar courses of infection and histologically evident liver disease as observed during HBV infection. The typical course of WHV infection, including the time of the appearance of viremia and development of virus-specific antibodies, may vary among animals inoculated with the same source and dose of virus (Michalak, 1998). Furthermore, the development of adaptive T cell proliferative responses and occurrence of liver injury may also vary (Menne et al., 1998). This suggests that yet unknown host factors are important, contributing to both virus expression and immune system-mediated clearance.

The first molecular indicator of WHV infection is the appearance of WHV DNA in the serum or peripheral blood mononuclear cells (PBMC) within 2 weeks p.i.

(Guo et al., 2000; Kajino et al., 1994; Menne et al., 1998; Michalak, 1998). WHV DNA has been identified in the liver as early as 2 weeks p.i. (Guo et al., 2000; Kajino et al., 1994), although earlier WHV DNA positivity cannot be excluded since such molecular analyses have not been performed. Interestingly, WHsAg and anti-WHc antibodies appear 4-10 weeks p.i., while antibodies to WHsAg preS1 domain may appear earlier, which co-incides with the appearance of WHV DNA in serum (Jin et al., 1996). In contrast, anti-WHs antibodies, mainly directed against the major S protein, do not normally appear until 10 weeks p.i. following clearance of WHsAg in those animals which resolve AH.

Since hepadnaviruses are non-cytolytic, liver disease is induced and perpetuated by the host's antiviral immune response and is characterized by similar histological changes as those identifiable during HBV infection. Proliferative responses of Kupffer cells and bile canaliculi are the earliest alterations which are followed by sinusoidal accumulations of lymphocytes and neutrophils occurring at 4-6 weeks p.i. This may be accompanied by sinusoidal endothelial cell proliferation. Liver architecture is progressively disrupted, concomitant with extensive leukocytic infiltrations and elevated serum levels of sorbitol dehydrogenase (SDH) indicative of liver injury (Menne et al., 1998). Clearance of WHsAg and the development of anti-WHs antibodies typically coincide with a significant decrease in lobular infiltrates while portal inflammation may persist. Indeed low grade inflammation persists for years following recovery from acute WHV infection (Hodgson and Michalak, 2001; Michalak, 1998).

Failure to clear WHV infection resulting in serum WHsAg-positive CH is characterized by portal and peri-portal infiltrates, piecemeal necrosis with focal parenchymal collapse, as well as proliferation of bile ducts and endothelial cells. WHV-induced HCC varies morphologically and different tumours may contain either differentiated hepatocytes or undifferentiated cells (Tennant et al., 2004). The mechanisms of HCC development likely include persistent hepatocyte regeneration and upregulation of anti-apoptotic regulatory proteins by the WHV X protein (Diao et al., 2001). In addition, DNA integration of the WHV genome, including promoter sequences, may lead to the activation of c-myc and n-myc oncogenes (Fourel et al., 1990; Hsu et al., 1990).

1.3.3.2.2 WHV-induced hepatitis

WHV infection has an overall pattern closely comparable to that of HBV infection (see Section 1.1.2.2) and is typically manifested as an acute, symptomatic, serum WHsAg-positive infection which is resolved in approximately 85-90% of animals. The progression to serum WHsAg-positive CH is observed in 10-15% of animals (Korba et al., 1989). More recent studies have demonstrated that recovery from AH is followed by indefinitely long persistence of low levels of replicating WHV in the liver and the lymphatic system, which is accompanied by small amounts of WHV DNA detectable in circulation (Michalak et al., 1999).

Recovery from WHV-induced hepatitis is associated with a higher hepatic virus load, a greater degree of liver injury and the augmented expression of interferon-gamma (IFN γ) and tumour necrosis factor-alpha (TNF α) (Hodgson and Michalak,

2001). Furthermore, WHV infection induces strong virus-specific T cell proliferative responses against epitopes of the virus envelope and core proteins (Menne et al., 1998). Interestingly, anti-viral T cell proliferative responses exhibit temporal differences characterized by the initial appearance of WHsAg-specific T cells followed by a T cell response of larger intensity directed against WHcAg (Menne et al., 1998), as similarly observed during HBV infection (Ferrari et al., 1990). Anti-viral T cells have been proven indispensable for viral clearance, since immune suppression using cyclosporin A prevents recovery and promotes development of CH (Cote et al., 1991).

While the peak intrahepatic occurrence of T cells coincides with the largest degree of liver injury (Guo et al., 2000; Hodgson and Michalak, 2001; Menne et al., 1998; Wang et al., 2004) observable during AH, anti-viral cytokines such as IFN γ and TNF α , appear to mediate significant noncytolytic clearance of hepadnaviruses (reviewed in (Guidotti and Chisari, 2001), and may play a greater role in the downregulation of hepadnaviral replication than the cytolytic removal of infected hepatocytes. IFN γ and TNF α are not only of paramount importance during AH (Hodgson and Michalak, 2001; Nakamura et al., 2001; Wang et al., 2003b), contributing to viral recovery, but their expression is sustained within the liver for years after resolution of AH in the presence of low levels of replicating WHV (Hodgson and Michalak, 2001). Although increased intrahepatic levels of these cytokines occur due to activation of virus-specific T cells, it has been noted that elevations in intrahepatic IFN γ can be detected before the onset of histologically evident hepatitis and T cell infiltrations (Hodgson and Michalak, 2001). Furthermore, increased cytolytic activity of innate immune cells (Hodgson et al., 1999) and decreases in intrahepatic viral loads

before the onset of evident hepatitis (Wang et al., 2004), are consistent with observations in the chimpanzee model of HBV which suggest a role for activation of innate immunity in hepadnaviral clearance and recovery (Guidotti et al., 1999).

As indicated, approximately 10-15% of adult animals infected with WHV progress to serum WHsAg-positive CH, which is a rate similar to that observed in adults infected with HBV. In contrast, WHV inoculation of woodchucks during the neonatal period may result in the development of CH with a frequency of 60-70% (Cote et al., 2000), as similarly observed during neonatal HBV infection of humans. Despite the delayed onset of viremia and histologically evident hepatitis following WHV infection of neonates (Cote et al., 2000), both neonatal and adult infections which progress to chronic disease exhibit similar defects in immune-mediated hepadnaviral clearance. The development of CH is associated with a diminished intrahepatic inflammatory response during the acute phase of WHV infection, characterized by a lesser degree of hepatic T cell infiltrates (Hodgson and Michalak, 2001; Nakamura et al., 2001; Wang et al., 2003b), decreased levels of IFN γ and TNF α (Hodgson and Michalak, 2001; Nakamura et al., 2001; Wang et al., 2003b), and increased viral load and antigen expression in the liver (Cote et al., 2000; Hodgson and Michalak, 2001) in comparison with a successful resolution of AH. In contrast with recovery from AH, where a strong, multispecific T cell response against several WHV antigens occurs (Menne et al., 1998), the development of chronicity is associated with a complete or partial absence of virus-specific T cell responses (Menne et al., 2002). These findings parallel those observed during CH type B (Ferrari et al., 1990; Maini et al., 2000; Penna et al., 1997). These results suggest that the development of CH is

largely attributed to a dysfunctional or otherwise lacking virus-specific cellular immune response (Hodgson and Michalak, 2001; Maini et al., 2000; Wang et al., 2003b) which may develop during a critical pre-symptomatic period (reviewed in (Webster and Bertoletti, 2002). Once established, chronic WHV infection presents several barriers to immune-mediated clearance including downregulation of class I major histocompatibility complex (MHC) protein display on the surface of infected hepatocytes (Michalak et al., 2000; Wang and Michalak, 2006) and the incorporation of virus envelope proteins into hepatocyte plasma membranes (Michalak and Churchill, 1988; Michalak and Lin, 1994).

1.3.3.2.3 Occult WHV infection

Hepadnaviral infection may also occur in the absence of symptoms, and remain serologically silent for the lifetime of the animal or individual (Michalak et al., 1999; Michalak et al., 1994; Penna et al., 1996). Clinical recovery from AH defined by normalization of biochemical liver function tests as well as seroconversion to anti-WHs antibodies does not truly reflect complete clearance of virus. Traces of WHV genomes and replicative intermediates persist in hepatocytes and cells of the lymphatic system and in the serum throughout life. Harboured virus persists despite both virus-specific humoral and cellular immunity (Michalak et al., 1999; Michalak et al., 1994; Penna et al., 1996).

The woodchuck model has also firmly demonstrated that vertical transmission of WHV occurs not only when the mother has serologically evident chronic WHV infection, but also after recovery from AH, during which time they carry low levels of

replicating virus (Coffin and Michalak, 1999). It is notable that WHV was consistently detectable in lymphoid cells in these offspring, while the liver was not universally infected. Furthermore, infection persisted in the complete absence of serological markers, including WHsAg and anti-WHc antibodies. Interestingly, in spite of low level viral infection, the offspring were not protected following challenge with a larger WHV infectious dose (Coffin and Michalak, 1999).

Although the pathological consequences of HBV infection to the infants born to HBsAg-positive, chronically infected mothers are widely recognized, the impact of vertical transmission of small quantities of the virus following maternal recovery from AH has not been studied. However, evidence from the woodchuck model suggests that vertically infected, asymptomatic and serologically undetectable, yet infectious HBV-positive carriers may represent a source of infectious HBV. Furthermore, long-term persistence of HBV in the lymphoid compartment may contribute to the induction of diseases not yet attributed to hepadnavirus infection. It is certain that animals which have recovered from acute WHV hepatitis continue to show mild to moderate hepatic inflammation for years following resolution of disease (Michalak et al., 1999). The most striking evidence of relevance of occult WHV persistence to pathology is the development of HCC in up to 20% of the recovered animals (Michalak et al., 1999).

1.4 ANTIVIRAL IMMUNITY

The immune system provides multi-layered protection against invasions with pathogenic microorganisms, including bacteria, parasites and viruses. Pathogens are initially recognized by soluble (naturally occurring IgM, complement proteins)

components of the innate immune system. Pathogen recognition subsequently leads to activation of cellular subsets (macrophage, dendritic cell [DC], natural killer [NK], natural killer T cell [NKT]), resulting in activation of anti-microbial processes including phagocytosis, production of anti-microbial agents (e.g., nitric oxide, granule-associated proteases) and secretion of immunomodulatory mediators (e.g., cytokines and chemokines). However, complete pathogen containment and removal requires activation of secondary immune defenses which includes pathogen-specific humoral and cellular responses.

In contrast to innate immune responses, adaptive immunity provides long lasting protection against microbial infection by antibody neutralization of extracellular pathogens, while cellular immune responses combat intracellularly replicating microbial agents. Experimental evidence indicates that innate immune cell subsets directly contribute to activation of adaptive immunity, and that significant cross-talk occurs between antigen presenting cells, NK and NKT cells, as well as $CD4^+$ T helper and $CD8^+$ cytotoxic T lymphocyte (CTL) effectors. Thus, host immunity is facilitated by complex cellular interactions, which not only provide anti-microbial protection, but which may also contribute to immune-mediated pathology, as is the case in virally induced hepatitis.

1.4.1 Innate immunity

The innate immune system provides the first line of defense against pathogens and relies upon a stepwise process which includes pathogen recognition and activation of cellular and soluble immune components. Briefly, the decision to respond or not to

respond to particular microbial antigens is mediated by innate recognition receptors which are invariable, germline encoded receptors expressed on innate immune cell subsets, including DC, macrophages, neutrophils (and other granulocytes), and NK and NKT cells. Each of these cell types may respond to infection with unique or partially overlapping effects, which may be mediated by direct cell-cell interactions, or via soluble mediators, which act upon other components of the innate or further downstream adaptive immune responses.

1.4.1.1 Antigen capture and presentation

Although the engagement and recognition of pathogens may elicit an immediate anti-microbial response, which is important for early containment, arguably the most crucial role for the innate immune responses is downstream activation of adaptive immunity. In this regard, highly specialized professional phagocytic cells mediate antigen recognition, uptake and presentation to T cells, facilitating subsequent antigen-specific responses.

DC represent the most highly specialized professional antigen presenting cells (APC). They may be derived from either myeloid or lymphoid (plasmacytoid) progenitors, and are further classified based upon the expression of various receptors, in addition to tissue specificity (reviewed in (Heath et al., 2004)). Although the functions of particular DC subsets may demonstrate considerable overlap, evidence does suggest that some DC types exhibit specialized immunological properties. Thus, plasmacytoid DC produce large amounts of type I IFNs (interferon α [IFN α] and interferon β [IFN β]) following contact with virions (Asselin-Paturel et al., 2001;

Kadowaki et al., 2001). The importance of this event is not fully understood, however, it may contribute to control of viral replication or to activation of other DC subsets (Diebold et al., 2003). For example, Langerhans' cells are a specialized DC subset resident within the skin epithelium which detect invading pathogens and subsequently migrate to draining lymph nodes where they prime T cell responses (reviewed in (Romani et al., 2003). Their involvement in activation of virus-specific T cells has been examined during HBV infection (Allan et al., 2003), and they appear to play a limited role in anti-HBV immunity.

Although DC have recently been identified as effective promoters of adaptive T cell responses, macrophages have long been recognized as immunologically important phagocytic cells. Circulating bone marrow-derived monocytes differentiate into macrophages following extravasation into peripheral tissues. Distinctly heterogeneous macrophage subsets, based upon expression of differentiation antigens and surface receptors, are localized within lymphoid organs and in non-lymphoid tissues, including the liver (Kupffer cells), lung (alveolar macrophages) and nervous system (microglia). Furthermore, monocyte recruitment and macrophage differentiation is observed during inflammatory reactions. Thus, anti-microbial immunity is mediated by both resident and newly recruited macrophages which contribute to pathogen clearance and containment, clearance of apoptotic and necrotic cells, and to tissue remodeling and repair (reviewed in (Mellman et al., 1998).

Pathogen recognition by the innate immune system can be facilitated by receptors (pathogen recognition receptors; PRR) expressed on the cell surface or within intracellular compartments, or by those which may also be secreted into circulation or

other bodily fluids. Soluble mediators of pathogen recognition may include mannan binding lectin (MBL) and c-reactive protein (CRP), both of which are produced by the liver during early acute phase responses (Fraser et al., 1998; Gewurz et al., 1982). Cell surface PRRs represent diverse protein families, each characterized by distinctive cellular distribution, capability of antigen binding and activation of downstream signaling and response elements (reviewed in (Taylor et al., 2005). Briefly, PRR families include scavenging receptors capable of bacterial or apoptotic cell recognition, complement or immunoglobulin receptors which recognize opsonized pathogens, and diverse groups of receptors which recognize microbial products, including lipopolysaccharide (LPS) (e.g., CD14) (Wright et al., 1990). More recently, it has been determined that a conserved family of Toll-like receptors (TLR) (Belvin and Anderson, 1996) is expressed by both humans and mice, and function to coordinate intracellular activation in response to pathogenic stimuli (reviewed in (Janeway and Medzhitov, 2002). TLRs transmit intracellular signals via a conserved mechanism which culminates in nuclear factor kappa B (NF κ B) activation and induction of phagocytosis, upregulation of costimulatory molecules necessary to induce adaptive immune responses, and production of inflammatory mediators important for the generation of T helper 1 (T_H1)-type immunity (Schnare et al., 2001).

1.4.1.2 Soluble components

1.4.1.2.1 Complement

The complement system represents not only an important mechanism for pathogen lysis and targeted removal by phagocytic cells, but also serves a more vital

role as a mediator of inflammation (reviewed in (Barrington et al., 2001)). Activation by pathogen recognition proteins such as MBL, CRP or immunoglobulin (particularly IgM) leads to microbial opsonization and injury. Furthermore, small inflammatory mediators generated by cleavage of complement components are instrumental for guiding extravasation of leukocytes and subsequent pathogen containment. Since the complement cascade is highly efficient and self-amplifying, all host cells express specific complement regulatory proteins which regulate its early activation (Hourcade et al., 1989). Deficiencies in complement regulation or aberrant complement activation may result in damage to host tissues. Hepadnaviral-induced production of autoantibodies to hepatocyte-specific asialoglycoprotein receptor (ASGPR) has been demonstrated to be pathologically relevant due to complement fixation (Diao et al., 1998). Furthermore, anti-ASGPR-directed complement-mediated hepatocellular injury modulates the severity of WHV-induced hepatitis (Diao et al., 2003). Notwithstanding its potential involvement in certain autoimmune conditions, complement activation supports adaptive immunity by: (1) localization of antigen to follicular dendritic cells which are important activators of humoral immunity (Papamichail et al., 1975), (2) lowering the threshold for antigen activation of naïve B cells (Carter and Fearon, 1992) while (3) supporting maintenance of long term memory B cells.

1.4.1.2.2 Nitric oxide

Nitric oxide (NO) was formally recognized as a tumoricidal and antimicrobial molecule which was produced by activated macrophages via the enzymatic activity of nitric oxide synthase (NOS), following microbial recognition (Nathan, 1992). It has

been subsequently determined that NO can be produced by many immune cell types, including APC such as DC or Kupffer cells, as well as granulocytes and NK cells. NO plays multiple roles in immune processes, including differentiation, proliferation, cytokine production and expression of costimulatory molecules (Bogdan, 2000). Although IFN γ and LPS are well characterized mediators of NO production, other activators include microbial products which stimulate TLR-dependent signaling, including bacterial lipoprotein, flagellin, or DNA (Thoma-Uszynski et al., 2001).

1.4.1.2.3 Immunomodulatory components

1.4.1.2.3a Chemokines

Various pathogens induce particular cytokine and chemokine responses depending upon the types of recognition receptors engaged. Although immunomodulatory soluble mediators are important effector molecules in innate activation, they also represent key intermediaries in the successful activation and polarization of adaptive immunity.

Chemokines comprise a complex communication system utilized not only by immune cells, but by all cell types, which mediates multiple intracellular signaling events. Chemokine responsiveness is determined by the levels of cell type specific expression of particular chemokine receptors (Murphy, 2002), which are partially redundant and overlapping. The major function of chemokines is leukocyte recruitment to sites of inflammation and regulation of trafficking through lymphoid organs (reviewed in (Rot, 1992)). The induction of chemokine expression following engagement of PRRs, or signaling potentiated by inflammatory mediators such as IFN γ

or TNF α (Baggiolini et al., 1997), is particularly important during innate immune responses since they encourage leukocyte recruitment and pathogen containment. Furthermore, chemokine signaling facilitates priming of adaptive immunity and homing of effector T cells into peripheral sites of infection (Weninger et al., 2001).

1.4.1.2.3b Interferons

Interferons are soluble factors which are capable of downregulating viral infection. Since their discovery, it has been determined that interferons represent two main types, namely type I and type II, as well as some interferon-like cytokines including IL-28A, IL-28B and IL-29 (reviewed in (Pestka et al., 2004). Type I IFNs currently represent seven classes which may be further subdivided into multiple isoforms, while type II interferon solely consists of IFN γ .

Type I IFNs are biologically active as monomers, each capable of binding a common IFN α/β receptor complex which mediates intracellular activation via several signaling cascades including JAK/STAT, MAPK and NF κ B pathways (reviewed in (Stark et al., 1998). Type I IFNs mediate antiviral activity by different mechanisms including induction of antiviral proteins, such as double-stranded RNA dependent protein kinase (PKR) which may degrade viral proteins, and 2'5'-oligoadenylate synthase (OAS) which selectively acts to degrade viral mRNA (Sen and Ransohoff, 1993). They are also important mediators of pathogen-specific immunity during non-viral infections including *Chlamydia* (Rothfuchs et al., 2001), *Leishmania* (Diefenbach et al., 1998), and *Trypanosoma* (Une et al., 2000). Briefly, IFN α/β affect DC function

(Santini et al., 2000), including antigen presentation and subsequent activation of T cells, B cells, and NK cell cytotoxicity (Nguyen et al., 2002).

IFN γ is a potent activator of DC and macrophages, and plays an essential role in resistance to many pathogens, including intracellular bacteria, parasites, and viruses. Although it was initially considered to be produced by activated T cells, the importance of IFN γ during innate immune responses has been highlighted by the observation that NK, NKT and phagocytes also produce large quantities of IFN γ (reviewed in (Lieberman and Hunter, 2002).

Not surprisingly, regulation of IFN γ is complex, however, one key regulator is the expression of heterodimeric interleukin-12 (IL-12) (Kobayashi et al., 1989), and its interaction with a dimeric receptor (IL12R) (Presky et al., 1996). DC production of IL-12 has been implicated as an important inducer of IFN γ during some intracellular infections or in response to LPS. Furthermore, bacterial or parasitic infections or viral replicative intermediates have been shown to induce IL-12 production and regulate T helper type I (T_H1) responses, including IFN γ production (reviewed in Trinchieri, 2003).

IL12R is expressed by activated T cells, however, it is also constitutively displayed on NK cells and may contribute to their ability to respond quickly to IL-12 with subsequent production of IFN γ (reviewed in Trinchieri, 2003). While IL-12 positively regulates NK cell production of IFN γ , artificial activation of NKT cells via synthetic glycolipids is an even stronger inducer of IFN γ . The importance of IFN γ in promotion of immune responses is multifaceted. NK cell-derived IFN γ activates

macrophages and promotes T cell independent maturation (Gazzinelli et al., 1994), while also activating non-immune cells, including naïve or virally infected hepatocytes.

1.4.1.2.3c Tumor necrosis factor alpha

TNF α is the prototypic member of the TNF superfamily whose members mediate diverse biological functions (reviewed in Locksley et al., 2001), including mediation of lymphoid organogenesis, inflammation and apoptosis. TNF α and other members exist both as membrane bound and soluble isoforms, the latter being generated by matrix metalloprotease-mediated cleavage. Membrane bound or soluble ligands may interact with several cognate receptors, which may themselves exist as membrane anchored or cleaved entities.

TNF receptors, including CD95 (Fas), TNFR1 or TNF-related apoptosis inducing ligand receptor (TRAIL-R) may potentiate both apoptotic and proliferative signals due to their interaction with various cytoplasmic signaling moieties. In this regard, caspase activation results in cellular apoptosis, while TNF-receptor associated factors (TRAFs) activate several proliferative pathways including NF κ B, JNK and PI3K (Dempsey et al., 2003). Thus, TNF superfamily member-mediated signaling is complex and dependent upon the type and activation state of the target cells (Hehlhans and Mannel, 2002).

TNF α -mediated signaling via TNFR1 is crucial for pathogen clearance including intracellular bacteria, such as *Listeria* (Pfeffer et al., 1993), and parasites including *Leishmania major* (Nashleanas et al., 1998). Furthermore, TNF α mediates protective T_H1 type responses, particularly in cooperation with IFN γ (Bekker et al.,

2001) during infection with *Mycobacterium tuberculosis*. The importance of TNF α as a stimulator of innate immunity is highlighted by cytokine-induced macrophage expression of NO and chemokine-dependent recruitment of inflammatory cells (Roach et al., 2002). Indeed, the action of TNF α on endothelial permeability is a key initiator of pathogen-induced inflammation, and promotes adaptive immunity by activation of phagocytic cells. Additional TNF receptors aid CD4⁺ and CD8⁺ T cell activation and are crucial for virus-specific T cell responses (Hendriks et al., 2000). The importance of TNF superfamily members in mediating antiviral immunity has been demonstrated by the evaluation of virus genes whose products inhibit TNF-mediated signaling at virtually every step, including expression, interference with ligand-receptor binding, and modulation of downstream pathways (reviewed in (Benedict et al., 2002).

1.4.1.3. Cellular Components

1.4.1.3.1 Neutrophils

Neutrophils are highly motile, phagocytic cells which are essential for immunity to bacterial and fungal infections. Their microcidal activity is attributed to fusion of phagocytic vacuoles with cytoplasmic granules containing hydrogen peroxide and superoxide radicals (Holmes et al., 1967), in addition to cytotoxic granule proteins such as lysozymes (Reeves et al., 2002). While initial works implicated reactive oxygen species and hydroxyl radicals to be key mediators of anti-microbial activity, it has since been determined that both free radicals and proteases are cooperatively required for effective microbial destruction (Reeves et al., 2002). Cytoplasmic granules typically fuse with endocytic phagosomes after the vacuolar membrane has

completely closed, to ensure that potentially damaging free radicals and anti-microbial proteins are not externalized. However, overwhelming excess of opsonized particles or inflammatory mediators may result in dysregulated degranulation. Neutrophil-mediated tissue injury has been implicated in the development of hepatitis in the HBV transgenic mouse system (Sitia et al., 2002). In this case, specific blockade of neutrophil recruitment and activation by interferons significantly reduced non-specific hepatocyte injury.

1.4.1.3.2 Natural killer cells

NK cells were initially identified through their ability to kill tumor cells without prior sensitization of the host to tumor-specific antigens, while subsequent studies shed light on their participation in the immune response to microbial infection (Bancroft, 1993). Further evidence has indicated that substantial cross-talk occurs between NK and other cell subsets of the innate immune response, including DC, resulting in the activation of these and other antigen presenting cells (Gerosa et al., 2002). The interaction of NK cells with APC may occur at sites of inflammation or within peripheral lymph nodes (Ferlazzo et al., 2004) and may result in IFN γ production or increased NK cytolytic activity (Yu et al., 2001). Reciprocal NK-DC activation likely involves both soluble mediators, such as interleukin-2 (IL-2) (Granucci et al., 2004) or TNF α (Gerosa et al., 2002), and direct cell contact (Piccioli et al., 2002).

Observations that NK cells displayed inherent capacity to kill certain tumor targets, which showed an inverse correlation between MHC class I expression and susceptibility to lysis (Karre et al., 1986) led to the “missing self hypothesis”. It is

presently recognized that inhibitory NK receptors, which belong to either killer Ig-like (KIR) or C-type lectin protein families, primarily engage host MHC class I molecules. Receptor cross-linkage mediated by host MHC molecules ensures dephosphorylation of proteins involved in cell activation, thereby conferring protection from NK cell attack (Long, 1999). Loss of MHC class I expression or absence of MHC-related molecules (e.g., MHC class Ib proteins) results in a lack of NK cell inhibition and subsequent default activation.

NK recognition additionally involves activation receptors, such as the recently identified NKp46 (Pessino et al., 1998), and NKp44. These receptors appear to mediate NK recognition and lysis of virally infected cells (Mandelboim et al., 2001), as well as certain melanoma, adenocarcinoma and hepatoma tumor cell types (Sivori et al., 1999).

Although cellular ligands and receptors mediating NK cell activation are not fully identified, it is apparent that NK cells are important immune effector cells during bacterial (Tripp et al., 1993) and parasitic infections (Scharton-Kersten and Sher, 1997). The involvement of NK cells in host immune responses to viral infection has been firmly established (reviewed in (French and Yokoyama, 2003)). NK cell contributions to containment of bacterial or parasitic infections, or to the generation of antiviral immunity, may involve either cytokine or cytolytic responses which depend upon the site of replication (Tay and Welsh, 1997), and the local cytokine milieu. In this regard, IFN α , IFN β , IL-12 and interleukin-15 (IL-15) produced by DC and macrophages (Biron et al., 1999) positively contribute to NK cell activation and cytokine secretion, and influence non-cytolytic adaptive immune responses (Guidotti

and Chisari, 2001). The importance of NK cells during viral infection is highlighted by the fact that many viruses have developed evasion strategies to specifically avoid NK cell recognition and activation. Herpes viruses, including human and murine cytomegaloviruses (CMV), downregulate cellular expression of ligands which bind the NK activation receptor, NKG2D (Lodoen et al., 2003). Furthermore, HCV envelope proteins have been shown to downregulate NK cell responses, particularly cytokine production (Pileri et al., 1998).

1.4.1.3.3 Natural killer T cells

NKT cells represent a heterogeneous lineage of thymically derived cells which are classified into four categories based upon expression of T cell receptor (TCR) variable regions and associated CD antigens (reviewed in (Kronenberg and Gapin, 2002). Although they constitute less than 1% of the T lymphocyte population, their ability to rapidly secrete cytokines, such as interleukin-4 (IL-4) and IFN γ , implies an important role in bridging innate and adaptive immunity. NKT cells exhibit diverse immunological functions, including prevention of tumor development and metastases (Smyth et al., 2002), amelioration of autoimmune and allergic diseases (Hong et al., 2001; Sharif et al., 2001), and activation of antiviral immunity (Ashkar and Rosenthal, 2003).

Since tumor or pathogen-derived ligands which activate NKT cells are not fully recognized, CD1d-restricted NKT cell function has been experimentally determined by applying the marine sponge-derived glycolipid, α -galactosylceramide (α -galcer) (Kawano et al., 1997). Thus, α -galcer-pulsed APC rapidly activate NKT-mediated

cytotoxicity (Metelitsa et al., 2001) or production of cytokines, including IL-4 and IFN γ (reviewed in (Bendelac et al., 1997).

The role of NKT cells in promoting viral immunity has not been fully elucidated. However, evidence from virus model systems including vaginal herpes simplex virus-2 (HSV-2) (Ashkar and Rosenthal, 2003) and respiratory syncytial virus (RSV) (Johnson et al., 2002), indicate that NKT cells activate APC and NK cells, and favor the development of virus-specific, T_H1-type T cell responses and the production of IFN γ . The involvement of NKT cells has been implicated during HCV infection, since HCV viraemic patients exhibit a lower frequency of invariant NKT cells (Lucas et al., 2003), while recovered individuals display an increased accumulation of activated NKT cells in the hepatic compartment. In addition, NKT cells were found to be selectively depleted during human immunodeficiency virus (HIV) infection, possibly contributing to the development of immunodeficiency (Motsinger et al., 2002).

The inherent capacity of NKT cells to produce IFN γ following α -galcer stimulation has been successfully shown to downregulate herpes virus replication in the absence of virus-specific T cell recognition, highlighting the potential therapeutic activation of NKT cells to facilitate viral clearance. Although a natural role for NKT in the downregulation of HBV is not recognized, α -galcer therapy in a HBV transgenic model leads to rapid NKT-associated IFN γ production, recruitment of NK and T cell subsets, and abrogation of viral replication (Kakimi et al., 2000).

1.4.2 Adaptive immunity

Following pathogen recognition by components of the innate immune system, the sequential and complementary activation of adaptive immune responses provides long lasting immunity. This immunity is conferred by effector and memory T cells, and by pathogen-specific antibodies. The interaction of innate immune cells with those of the adaptive immune response is not, however, unidirectional. Rather, experimental evidence demonstrates the importance of activated T cells in the reciprocal activation of innate immune cells, including APC.

1.4.2.1 Humoral immune responses

Humoral immunity mediates protection against extracellular microbes by specific antibodies. These antibodies are induced following engagement of surface immunoglobulin expressed on naïve B cells and their development into antibody producing plasma cells. Antibodies may contribute to pathogen inactivation and clearance by several mechanisms. Neutralizing antibodies prevent viral attachment and entry into host cells and may act as opsonins which promote phagocytosis. Furthermore, antibodies efficiently activate the complement cascade, particularly those of IgM or IgG subclasses. Natural IgM antibodies, which are produced by CD5-expressing B cells, contribute to the initiation of innate immunity to some pathogens via complement fixation and subsequent chemoattraction of phagocytic cells. T helper 2 (T_H2)-type CD4⁺ T cells promote activation of B cells and production of pathogen-specific antibodies, which may also initiate the complement cascade. Complement fixation mediated by antibody recognition of viral antigens on host cells facilitates

CDC, while antibody-dependent cellular cytotoxicity (ADCC) is potentiated by the interaction of bound immunoglobulin constant regions (F_C) with cellular F_C receptors.

1.4.2.2 Cellular responses

$CD4^+$ T_H cell responses are initiated by MHC class II-restricted presentation of exogenously acquired antigens (Cresswell, 1996). Cell-mediated T_H cell responses are divided into two categories, T_H1 and T_H2 . The main determining factor in the polarization of the type of T_H responses is the cytokine milieu present during the clonal expansion of the specific T cells (Mosmann and Coffman, 1989). Thus, the development of T_H1 or T_H2 responses are facilitated by exposure to IL-12 (Kobayashi et al., 1989) or IL-4 (Le Gros et al., 1990), respectively.

A T_H1 cell response is characterized by the production of cytokines including IL-2 and $IFN\gamma$, and is often associated with the development of $CD8^+$ T cell immunity. $CD4^+$ T cells have been shown to provide co-stimulatory activity required for maturation of APC and subsequent presentation of antigens to $CD8^+$ T cells (Bennett et al., 1997). In the absence of maturation signals provided by $CD4^+$ T cells, APC may induce $CD8^+$ T cell tolerance and inactivation (Curtsinger et al., 2003). In addition, lack of $CD4^+$ T cell help during the development of $CD8^+$ T cell adaptive immunity results in impaired development of memory T cells, and lack of protective immunity to viral and intracellular bacterial infections (Sun and Bevan, 2003). In contrast, T_H2 -type cell responses are characterized by $CD4^+$ T cells which produce cytokines including IL-4 and interleukin-10 (IL-10), which induce phenotypic and functional maturation of B lymphocytes.

CD8⁺ T lymphocytes are important immune effector cells against intracellular replicating bacterial, protozoan and viral pathogens. Their activation requires presentation of microbial peptides within the context of MHC class I molecules. Generation of such peptides occurs via cytosolic, proteasome-dependent degradation of proteins which are subsequently retrogradely transported into the ER where they associate with MHC class I heterodimers. Professional APC, such as DCs, are able to cross-present exogenous antigens obtained by phagocytic activity via the MHC class I pathway, thus possibly precluding the necessity of direct pathogenic infection of the DC for CD8⁺ T cell activation (reviewed in Heath et al., 2004). Since MHC class I molecules are expressed on essentially all host cells, primed CD8⁺ CTL may respond against cellular pathogens infecting most tissues. CTL effector functions are mediated by direct cell contact and cytolysis, or by soluble mediators including IFN γ and TNF α .

CTL-mediated lysis of target cells may proceed via two distinct yet potentially overlapping pathways; the granule exocytosis pathway mediated by perforin and granzymes, or by CD95L (Fas ligand)-CD95 (Fas) interaction, which can initiate apoptosis of CD95-expressing cells (reviewed in Barry and Bleackley, 2002). CTL-mediated cytotoxicity significantly contributes to removal of virally-infected cells (Kagi et al., 1994) and clearance of intracellular bacteria, including *Listeria* and *Chlamydia* (Starnbach et al., 1994).

In addition to mediating cytotoxicity, CD8⁺ T cells produce proinflammatory and antiviral cytokines, such as IFN γ and TNF α . These cytokines have been proven essential for successful clearance of WHV (Hodgson and Michalak, 2001; Wang et al., 2003b) and HBV infections (Guidotti et al., 1996; Guidotti et al., 1999).

1.5 THE LIVER AS AN IMMUNE COMPETENT ORGAN

The immune system is functionally organized based on the primary lymphoid tissues, thymus and bone marrow, and on peripheral secondary lymphoid organs, including lymph nodes, spleen, and mucosal-associated lymphoid tissues. The importance of primary and secondary lymphoid tissues in the initial development and selection of lymphoid cells, and the potentiation of immune responses, respectively, have been intensely studied. However, the role of the liver as a lymphoid organ is an emerging concept based upon clinical and experimental evidence. The observation that the liver is inherently capable of tolerance induction following solid organ transplantation was made more than 30 years ago (Calne et al., 1969) and has since been repeatedly supported by many observations (see Section 1.5.3.3). The mechanism of hepatic induced tolerance during organ transplantation and to dietary food antigens are a function of: (1) immunomodulatory properties of hepatic parenchymal cells, (2) its unique composition of immune cells, and (3) the microvascular structure of the liver. The complex interactions between lymphoid cells and APC following antigen recognition within the liver are important not only during tolerance induction but may contribute to pathogen-specific immune responses. In this regard, the interaction between hepatic parenchymal and local immune cells may contribute to clearance or persistence following infection with hepatotropic viruses. In addition to tolerance induction, other studies suggest that hepatic functions may include induction of primary T cell responses or active removal of effector T lymphocytes. This section will summarize the current understanding of the function of the liver as an immune competent organ.

1.5.1 Hepatic microenvironment

The liver displays excretory, detoxifying and metabolic functions. It is located between the gastrointestinal portal tract and systemic blood circulations. Approximately 80% of the hepatic blood supply enters via the portal vein, which collects blood from the gut (from the stomach to the proximal part of the rectum) that is rich in food antigens, environmental toxins and bacterial products including LPS. Additionally, all venous blood from the spleen enters into the portal vein. Thus, all recirculating naïve and activated or memory lymphocytes leaving the spleen are inevitably confronted with the specialized microenvironment of the liver. The remaining 20% of the incoming blood is supplied by the hepatic artery which mixes with the portal blood within the liver sinusoidal spaces. Blood percolates through a network of sinusoids which lack a basement membrane but are formed by liver sinusoidal endothelial cells (LSEC). Blood is finally directed into terminal central veins before entering systemic circulation via the inferior vena cava.

LSEC are highly fenestrated, allowing macromolecules to enter the underlying space of Disse and to directly interact with hepatocytes. Endothelial fenestrations and a lack of intercellular LSEC gap junctions also permit contact between hepatocytes and cells residing in or circulating through the sinusoidal spaces (Warren et al., 2006), or which enter into the space of Disse. The combination of slow blood flow, fenestrated endothelium, and lack of basement membrane facilitates a unique vascular microenvironment which contributes to intrahepatic immunological functions (see below).

1.5.2 Components of the intrahepatic immune system

1.5.2.1 Professional antigen presenting cells

The liver contains a large population of resident macrophages known as Kupffer cells. Although they are mostly found within the hepatic sinusoids, they may also penetrate into the space of Disse and interact directly with hepatocytes. They are highly phagocytic, capable of endocytosis of apoptotic cells (Shi et al., 1996) and microbial pathogens.

Hepatic tissue contains multiple subsets of DC (see Section 1.4.1.1) which are known to efficiently activate T cell responses, including proliferation and the induction of T cell cytotoxicity. DC have been shown to interact with Kupffer cells (Uwatoku et al., 2001), facilitating enhanced cross-presentation of exogenously acquired antigens and priming of anti-microbial responses (Albert et al., 1998).

In addition to professional APC, other studies indicate that LSEC may function in an antigen-presenting capacity. LSEC express molecules that promote antigen uptake, including the mannose receptor and scavenging receptor. Furthermore, the co-stimulatory molecules required for T cell activation, including CD40, CD80 and CD86 are readily detectable on LSEC (Lohse et al., 1996).

1.5.2.2 NK and NKT cells

Liver-associated NK cells have been initially identified as "pit cells" and exhibit a phenotype of large granular lymphocytes displaying cytotoxic activity against various cell targets, including YAC1 lymphoma cells (Bouwens et al., 1987). Liver-derived NK cells express similar activation and inhibitory receptors as those observed

for circulating NK cells. However, NK cells are of a disproportionately high frequency (31%) among intrahepatic lymphocytes (IHL) compared with the extrahepatic compartment (13%). Experimental evidence indicates that hepatic NK cells proliferate in response to antigenic stimulation, including viral infections and may be recruited into the liver in response to type I and type II IFNs (reviewed in (Biron et al., 1999)).

While NKT cells represent <5% of peripheral lymphocytes, they represent 20-40% of IHL (Klugewitz et al., 2004). Hepatic NKT cells may be activated independently of T cell receptor (TCR) engagement. For example, IL-12 (Hashimoto et al., 1995) or interleukin-18 (IL-18) (Leite-De-Moraes et al., 1999) induce NKT activation and production of IFN γ . In contrast with NK cells, activated NKT cells do not undergo clonal expansion and differentiation. Rather, they undergo activation induced cell death (AICD) within hours after stimulation with either IL-12 or anti-CD3 antibodies (Eberl and MacDonald, 1998), or during infection with *Listeria* (Szalay et al., 1999) or HCV (Nuti et al., 1998). Thus, efficient priming of intrahepatic T_H1 responses may coincide with activation of NKT cells, reflected by IFN γ production, and their subsequent transient depletion.

1.5.2.3 Conventional T cell subsets

In addition to disproportionate numbers of NK and NKT cells, the liver differs significantly in the phenotypic and functional properties of conventional T cells, expressing α and β chains of the TCR (denoted $\alpha\beta$ T cells), when compared with those in peripheral circulation (reviewed in (Doherty and O'Farrelly, 2000)). Less than 40% of IHL are conventional $\alpha\beta$ CD4⁺ or CD8⁺ T lymphocytes, while similar subsets

represent 70% of circulating T cells. Among $\alpha\beta$ T cells, those expressing the CD8 co-receptor predominate, comprising between 60-90% of conventional hepatic T cells. Although CD8⁺ T cells represent the majority of conventional T cells within the liver, CD4⁺ T cells are also detectable and tend to exhibit a T_H1 phenotype characterized by production of cytokines including IL-2 and IFN γ .

1.5.2.4 Non-conventional T cell subsets

The liver also contains T cell subsets which are atypical, including those which are CD4 and CD8 double-negative or double-positive, those that express homodimeric CD8 α chain in the absence of the CD8 β chain, and those whose TCR is comprised of the alternate $\gamma\delta$ chains. Up to 35% of hepatic T cells express the $\gamma\delta$ TCR and, unlike conventional T cells, they are believed to be of extrahepatic origin. Hepatic $\gamma\delta$ T cells support intrahepatic priming of T_H1-type responses to bacterial infections, and contribute to liver injury via cytotoxic mechanisms (Ishigami, 1999). However, other evidence suggests their involvement in skewing T cell responses towards an immunosuppressive T_H2 phenotype during the early phase of *Leishmania* infection (Yamashita et al., 1999). Thus, the contribution of hepatic $\gamma\delta$ T cells to immunity may include either T_H1 or T_H2 responses depending upon the pathogen encountered.

1.5.2.5 Parenchymal and endothelial cell contribution to immunity

LSEC are able to present exogenous antigens to T cells, since they constitutively express MHC class I and class II molecules, as well as co-stimulatory molecules (Lohse et al., 1996). They may even cross-present exogenous antigens to

CD8⁺ T cells, a function which is normally an attribute of professional APC (Limmer et al., 2000). However, LSEC-primed CD4⁺ T cells fail to differentiate towards a T_H1 phenotype, but rather secrete cytokines associated with T_H2-type responses, including IL-4 and IL-10 (Knolle and Gerken, 2000). In support of this, stimulation of naïve CD8⁺ T cells by LSEC results in transient proliferation but failure to acquire cytotoxic effector function (Bertolino et al., 2001; Limmer et al., 2000). Thus, the role of LSEC in the generation of immunity appears to be, by default, induction of CD8⁺ T cell tolerance and prevention of inflammation. It has been speculated that this default pathway is essential for maintaining immune ignorance of dietary and neoantigens (reviewed in (Knolle and Limmer, 2001). However, pathogen-induced inflammatory reactions including those characterized by TNF α production, downregulate antigen presentation by LSEC and promote T_H1-type cellular immunity (Knolle et al., 1999).

Both LSEC and hepatocytes express intercellular-adhesion molecule 1 (ICAM-1) which binds integrins expressed by activated T cells, including lymphocyte function antigen 1 (LFA-1). It has subsequently been shown that T cells may bind to hepatocytes (Morita et al., 1994). Furthermore, hepatocytes constitutively express MHC class I and CD1 molecules, albeit at low levels. Notwithstanding the minimal expression of these ligands, hepatocytes are able to induce CD8⁺ T cell activation *in vitro* (Bertolino et al., 1998). Additionally, it has been shown that inflammatory cytokines enhance the ability of hepatocytes to act as APC *in vivo*, due to upregulation of ICAM-1, MHC class I and class II, and co-stimulatory molecules (Bertolino et al., 2001).

1.5.3 Immunological function of the liver

1.5.3.1 Anti-microbial effector function

The contribution of hepatocytes to anti-microbial immunity is associated with activation of innate immune cell subsets within the liver. The recognition of microbial pathogens by macrophages and other cells, result in the production of cytokines, such as $\text{TNF}\alpha$, interleukin-1 (IL-1) and interleukin-6 (IL-6), which induce hepatocyte expression of acute phase proteins, including CRP and MBL. Both of these compounds are important for control of bacterial infections and aid in the development of adaptive immune responses (Fraser et al., 1998).

Since the majority of the hepatic blood supply is obtained from intestinal tissues, intrahepatic immune subsets readily respond to enteric pathogens which may penetrate the intestinal mucosa and enter systemic circulation. LPS, a cell wall component of Gram-negative bacteria, activates Kupffer cells resulting in the production of proinflammatory $\text{T}_\text{H}1$ cytokines (Trinchieri, 1998). NK and NKT cells may respond directly to superantigens of Gram-positive bacteria, including *Staphylococcal* enterotoxin B (SEB), by producing both $\text{IFN}\gamma$ and IL-12. Thus, entry of pathogenic bacteria into the hepatic sinusoids induces local anti-microbial responses.

Activation of intrahepatic immunity during infection with *Plasmodium berghei* is important for parasite clearance and protective immunity. In this regard, the interaction between $\text{IFN}\gamma$ -producing CD8^+ T cells and Kupffer cells, leading to production of NO, is a key factor providing protection against the exoerythrocytic stages of malaria (Seguin et al., 1994). Furthermore, activation of hepatic NK and

NKT cells appears to be required for development of long term memory CTLs and protective T_H1 -type immunity (reviewed in (Krzych et al., 2000)).

1.5.3.2 Lymphocyte retention and removal

Activation of T cells leads to clonal expansion and their migration to sites of infection, before their homeostatic downregulation. It has been noted that following activation by antigenic peptide (Mehal et al., 2001) or due to extrahepatic viral infection (Belz et al., 1998) there is a transient accumulation of T cells within the liver. While antigen presentation by either LSEC or hepatocytes may increase antigen-specific T cell retention (Mehal et al., 2001), it is not a prerequisite for hepatic T cell trapping (Belz et al., 1998). The accumulation of activated lymphocytes predominantly involves $CD8^+$ T cells, which are subsequently removed by the induction of apoptosis (Mehal et al., 1999).

Hepatic accumulation of T cells could be a consequence of interactions between adhesion molecules expressed by liver endothelial cells or hepatocytes and their ligands which are increasingly expressed on activated lymphocytes. In this regard, LSEC and hepatocytes constitutively express ICAM-1 which is capable of binding LFA-1 (reviewed in (Knolle and Gerken, 2000)). Activated $CD8^+$ T cells express higher levels of LFA-1 than $CD4^+$ T cells (Mehal et al., 1999) which may partially explain preferential $CD8^+$ T cell retention by the liver.

It has also been suggested that the retention of T cells within the liver may be facilitated by recognition of apoptotic cells. The concept proposes that the liver functions as a graveyard for dying T cells (reviewed in (Crispe et al., 2000)). Kupffer

cells are capable of recognizing apoptotic cells via the phosphatidyl serine receptor (Dini, 2000). It has been found that injection of apoptotic lymphocytes into the portal vein results in their retention, which is specifically inhibited following saturation of scavenging receptors (Dini, 1998). It has been established that hepatocytes may recognize cells or apoptotic bodies following interaction of the primarily hepatocyte-specific ASGPR with desialylated glycoproteins (Dini et al., 1992). This interaction has been suggested to play a role in the liver sequestration of lymphocytes (Samlowski et al., 1984) which may be due to the expression of ligands capable of binding to ASGPR (e.g., B220) following lymphocyte activation (Renno et al., 1996).

However, hepatic retention of T cells is unimpaired in CD95-deficient mice (Mehal and Crispe, 1998) and thus, trapped cells need not be apoptotic. This may suggest that the liver is responsible for initiation of lymphocyte apoptosis. Current evidence suggests that this may occur via both active and passive cell death mechanisms. Active cell death is induced by ligation of a death receptor (e.g., CD95) with its specific ligand (e.g., CD95L), while passive cell death is induced by withdrawal of growth factors or inhibition of activation signals. Identification of the pathways mediating intrahepatic lymphocyte removal has proven so far to be contradictory. Limmer et al. (2000) suggest that antigen presentation is necessary for CD95-dependent apoptotic signaling, while others report that T cells are trapped independent of antigen recognition (Mehal et al., 2001) and undergo CD95-independent deletion (Mehal and Crispe, 1998). Although the study conducted by Mehal et al. (1998) did not indicate which alternate mechanisms are potentially responsible, other works suggest that TNF α (Weishaupt et al., 2000) or perforin-dependent pathways mediate T

cell protraction (Kagi et al., 1994). Nonetheless, the roles of these pathways in hepatic removal of lymphocytes are not fully recognized.

Passive apoptosis of intrahepatic lymphocytes has also been observed and may be mediated by several mechanisms. It has been suggested that a lack of IL-2 (Dai et al., 1999) or presence of transforming growth factor beta (TGF β) and IL-10 (Sillett et al., 2001) promote passive cell death of CD8⁺ T cells. Of note, Kupffer cells (Rai et al., 1997) and LSEC (Bissell et al., 1995) produce large amounts of these cytokines which have been shown negatively influence the survival of CD8⁺ T cells, particularly when CD4⁺ T cells are absent. Since the liver preferentially retains CD8⁺ T cells, these observations suggest that the intrahepatic environment may promote passive CD8⁺ T cell deletion.

1.5.3.3 Induction of tolerance

The maintenance of allogeneic liver grafts without the need for immunosuppression was initially identified in pigs (Calne et al., 1969). Subsequent studies in rats (Zimmermann et al., 1979), mice (Qian et al., 1994), and humans (Eddleston et al., 1969) have demonstrated similar tolerogenic states. This may be due to multiple factors, including intrahepatic elimination of autoreactive T cells or the unique intrahepatic cytokine milieu which does not support T_H1-type cellular immune responses (Tu et al., 1997). Further, it has been found that the liver constitutively induces tolerance to food antigens (reviewed in (Knolle and Gerken, 2000) and may provide an anti-inflammatory environment allowing escape of metastatic tumour cells (Jewell, 2005). Thus, the inherently tolerogenic capacity of the liver may prevent

aberrant immune responses or be detrimental for host recognition of malignant tissues, while the mechanisms of tolerance induction seem to overlap with those of removal of activated lymphocytes trapped in the liver.

Although intrahepatic trapping of activated T cells preferentially involves CD8⁺ CTLs, tolerance induction has been observed to include both CD4⁺ and CD8⁺ T cell subsets. However, the mechanisms appear to differ; tolerization of CD4⁺ T cells involves immune deviation towards a T_H2-type response (Knolle et al., 1999), while CD8⁺ T cell activation is abrogated by deletion or immunological ignorance (reviewed in (Bertolino et al., 2002).

1.5.3.4 Induction of primary T cell immune responses

The data outlined so far implicate an important immunological role of the liver in removal of activated CTLs which may downregulate these cellular immune responses. At the same time, the liver also displays an inherent capacity to limit unwanted inflammatory responses to innocuous dietary antigens, which appears to occur primarily due to the production of anti-inflammatory cytokines such as IL-10 by LSEC and other intrahepatic APC (Knolle et al., 1999). It is not fully understood how the liver overcomes this default state of tolerance to allow activation of naïve T cells, although this phenomenon has been observed.

Although the exact mechanisms remain unknown, it is possible that intrahepatic priming of T cells following viral challenge, including infections with hepadnaviruses, may be mediated by the induction of type I IFN expression. Type I IFNs induce production of cytokines, including IL-15, which may promote survival of CD8⁺ T cells

(Mattei et al., 2001). Furthermore, activation of intrahepatic DCs and the downregulation of LSEC antigen presenting function may shift the balance from tolerance to immunity (Knolle and Limmer, 2001). Since hepatic DC are maintained in a semi-mature state and tend to promote CD4⁺ T cell regulatory function (O'Connell et al., 2002), the activation of innate cytokine responses, particularly IFN $\alpha\beta$, may promote full DC maturation (Luft et al., 1998) and lead to effective priming of effector T cells.

1.6 IMMUNOPATHOGENESIS OF HEPADNAVIRAL INFECTION

1.6.1 Innate immune response

The incubation period of HBV and other hepadnaviruses is long in comparison with other hepatotropic viruses such as HCV. While viraemia is detectable in cases of HCV infection within 1-2 weeks (Thimme et al., 2001), HBV infection is asymptomatic for 4-7 weeks or up to 6 months, until commencement of vigorous replication and development of AH (Whalley et al., 2001). Whether this delay represents an inherent property of hepadnaviruses or if early viral replication is decreased by immune control, is still unrecognized.

Initial viral infection and immediate activation of innate immune components are believed to influence the outcome of hepadnaviral infection, i.e., recovery or progression to CH. Although a complete assessment of immune activation preceding acute liver injury is currently lacking, experimental infection of chimpanzees suggests that hepatic HBV replication is significantly reduced prior to the development of liver injury characterized by elevations in the levels of serum aminotransferases (Guidotti et al., 1999). Further studies have indicated that activation of relatively few numbers of

IFN γ -producing CD8⁺ T cells is sufficient to downregulate viral replicative intermediates, as much as 50-fold, and that this occurs before the development of AH (Wieland et al., 2004b).

It has been reported that hepadnaviral-induced AH is associated with enhanced NK cell cytotoxicity both in humans (Chemello et al., 1986) and, potentially, in woodchucks infected with WHV (Hodgson et al., 1999). Furthermore, it has been demonstrated using the HBV transgenic mouse model that activation of NKT cells by α -galcer, with possible bystander involvement of NK cells, significantly downregulates HBV replication in an IFN γ -dependent manner (Kakimi et al., 2000). In addition, it has been shown that α -galcer-induced inhibition of viral replication does not require the recruitment of other inflammatory cells to the liver (Kakimi et al., 2001). Whether intrahepatic innate immune subsets, including NK and NKT cells, are immunologically active during the pre-acute phase of natural hepadnaviral infection, is not fully recognized. However, experimental evidence obtained thus far indicate that for antiviral responses to be effective, they should include the production of IFN γ , TNF α and IFN $\alpha\beta$ (Guidotti et al., 1994; Guidotti et al., 1996; McClary et al., 2000).

1.6.2 Humoral immunity

The humoral anti-hepadnavirus response appears to be of paramount importance for viral neutralization and recovery. Specifically, the loss of serum HBsAg and seroconversion to anti-HBs antibodies is the hallmark of disease resolution signifying recovery from AH type B, while CH is characterized by the continuous presence of serum HBsAg. However, even in chronic HBV infection anti-HBs may not

be completely lacking and they could be found complexed with HBsAg and deposited both in hepatic tissues (Nowoslawski et al., 1972) as well as in extrahepatic locations (see Section 1.1.4).

The importance of antibody responses against HBcAg and HBeAg in the pathogenicity of liver injury during CH are less clear. However, seroconversion to anti-HBe seems to represent a good prognostic indicator and a marker of recovery (Hoofnagle, 1981), possibly as a result of antibody-mediated clearance of HBe-reactive hepatocytes (Schlicht et al., 1991). It has been suggested that CDC mediated by anti-HBc and/or anti-HBe and directed against hepatocytes which express the respective antigens may contribute to liver injury particularly in cases of severe CH (Michalak et al., 1995).

Antibody responses against HBV polymerase and X proteins are not clinically monitored, however they may be reflective of HBV replication (Weimer et al., 1990). They are detectable at the highest levels in chronically infected patients while patients with AH and asymptomatic carriers may lack anti-HBx reactivities (Stemler et al., 1990).

1.6.3 Cell-mediated immune responses

Although our understanding of whether innate immune components contribute to hepadnaviral clearance is still poorly recognized, it is well known that a multispecific, polyclonal and vigorous T_H1 $CD4^+$ T cell and $CD8^+$ CTL responses are necessary for successful viral downregulation and recovery from hepatitis. $CD4^+$ T cells exhibit strong proliferative reactivity against several HBV antigens, regardless of

the patients' MHC haplotype (Ferrari et al., 1991). In agreement with the findings implicating the importance of IFN γ and TNF α cytokines for controlling viral replication, the appearance of CD4⁺ T cell responses during AH are found to produce high levels of T_H1 cytokines and, therefore, likely promote efficient activation of virus-specific CTL (Penna et al., 1997). The importance of CD4⁺ T cell responses to the control of hepadnaviral infection is highlighted by the long-term persistence of HBV-specific CD4⁺ T cells identifiable in PBMC derived from patients convalescent from AH type B (Rehermann et al., 1995a). Since HBV DNA can be persistently detected many years following recovery, when sensitive PCR combined with Southern blot hybridization analysis of the amplified viral DNA are used (Michalak et al., 1994), the peripheral HBV-specific CD4⁺ T cell pool is possibly maintained by low levels of replicating virus (Rehermann et al., 1996).

Although CD4⁺ T cell responses are important mediators of hepadnaviral clearance, primarily via the production of T_H1 cytokines, the functionality of virus-specific CTLs is of critical importance since they recognize virally infected hepatocytes following TCR ligation with MHC class I molecules presenting viral epitopes (see Section 1.4.2.2). A vigorous, polyclonal CTL response against multiple HBV epitopes can be identified during AH and, similarly to CD4⁺ T cell responses, the polyclonality and multispecificity persists for decades following serological recovery from AH (Rehermann et al., 1996). Using the transgenic mouse model of HBV, it has been determined that CTLs may contribute to non-cytolytic control of hepadnaviral replication (Guidotti et al., 1996), while also contributing to hepatocyte destruction by both CD95L and perforin-dependent mechanisms (Nakamoto et al., 1997).

In contrast to those CD4⁺ T cell responses observed during AH, the HBV-specific CD4⁺ T cell responses in patients with CH are much weaker and more narrowly focused (Ferrari et al., 1990). Furthermore, these cells do not efficiently produce IFN γ (Schlaak et al., 1999). Similarly, the peripheral CTL response to HBV antigens is weak or undetectable (Rehermann et al., 1995b). Recent works indicate that the intrahepatic CTL response is not completely lacking, as previously thought. Using MHC class I tetramers, which identify T cell reactivity based upon the combination of MHC and peptide recognition (reviewed in (Ogg and McMichael, 1998)), it has been shown that CH type B is in fact associated with low, although evident intrahepatic CTL responsiveness (Maini et al., 2000). The reasons for this remain unclear, however CD8⁺ T cell impairment has also been observed in cases of AH when viral loads were high (Maini et al., 1999). In cases of CH, liver injury is not due to virus-specific CTL but has been attributed to bystander recruitment and activation of non-virus-specific CD8⁺ T cells (Maini et al., 2000). Specifically, the frequency of circulating, or liver-infiltrating CD8⁺ T cells specific for the 18 – 27 peptide of HBcAg did not differ between chronically-infected patients displaying low or elevated serum concentrations of ALT, reflective of liver injury. Further, it was demonstrated that patients which exhibited severe hepatic infiltrations and increased levels of ALT displayed enhanced recruitment of CD8⁺ T cells which were not reactive to viral antigens, suggesting that non-specific CTL contributed to liver injury without control of viral replication (Maini et al., 2000).

1.7 PURPOSE OF THE STUDY

Recent evidence suggests that the liver is *de facto* an immune competent organ with distinct immunological functions. The role of the liver in host immunity is likely a consequence of interplay between hepatic parenchymal cells, including hepatocytes, sinusoidal endothelial cells and resident immune cells, and the immune cells trafficking through the liver. The unique hepatic microvascular structure, characterized by the absence of a basement membrane and highly fenestrated endothelium, facilitates these cellular interactions. It has been shown that the liver sinusoidal epithelium and hepatocytes display an inherent capacity to initiate as well as to inhibit adaptive T cell responses. These unique properties may be crucial for promoting both local and systemic pathogen-specific immunity and for maintaining peripheral tolerance against hepatotropic pathogens.

The liver contains a disproportionably high number of innate immune effector cells, which are important initiators of specific immune responses. Their presence may contribute to the local pathogen containment, as well as to liver injury in infections caused by noncytopathic hepatotropic viruses. Experimental evidence provided by studies in the HBV transgenic mice implicates the potential involvement of innate immune cells in the non-cytopathic, IFN γ -mediated downregulation of HBV replication. On the other hand, hepatocellular injury, which is a hallmark of viral hepatitis, has been attributed to response from both innate and adaptive immune cell subsets. Nonetheless, a contribution of the innate immune response to elimination of hepadnavirus and to activation of adaptive anti-viral immunity has not been thoroughly analyzed during progression of natural hepadnaviral infection, particularly in its early

phase which appears to be critical in determining the outcome of hepadnaviral hepatitis.

Experimental infection of adult woodchucks with WHV represents the closest virological and immunopathogenic model of human hepatitis B. This model was utilized in the current series of our studies. The objectives of these studies were:

1. To investigate the mechanism(s) by which hepatocytes may directly contribute to local liver immune responses, hepatic tissue injury or the downregulation of immune responses. Using primary freshly isolated hepatocytes as well as cultured hepatocytes and liver-derived hepatoma cell lines, to examine whether hepatocytes may mediate CD95L- or perforin-dependent cytotoxicity against heterologous cell targets. Also, to determine if pro-inflammatory and anti-viral cytokines, such as IFN γ or TNF α , may influence the cytotoxic potential of hepatocytes *in vitro*.
2. To determine if hepadnaviral infection influences the capacity of hepatocytes to act as cytotoxic effector cells via CD95L- and/or perforin-dependent pathways. For this purpose, primary hepatocytes will be isolated from woodchucks with experimentally induced resolved AH or chronic WHV hepatitis, as well as from healthy animals as controls, and their cell killing potential will be evaluated in *ex vivo* cytotoxicity assays directed against heterologous CD95-sensitive or CD95-insensitive cell targets. Similar analyses, using cultured woodchuck hepatocytes stably transfected with complete WHV genomes or individual

WHV genes, will be used to investigate whether hepadnavirus or its individual translation products may directly modulate hepatocyte cytotoxicity.

3. To determine early events occurring in the liver immediately after invasion with hepadnavirus and in the pre-acute phase of hepatitis. In particular, to examine whether experimental WHV infection activates intrahepatic innate immunity. In this regard, quantification of viral genomes and their replicative intermediates, as well as analysis of expression of innate and adaptive immune cell markers, cytokines and cell cytotoxicity indicators in liver tissue samples serially collected as early as one hour post infection and throughout the course of acute WHV-induced hepatitis will be performed.

CHAPTER TWO: HEPATOCYTES AS CYTOTOXIC EFFECTOR CELLS CAN INDUCE CELL DEATH BY CD95 LIGAND-MEDIATED PATHWAY

This study has been published in Hepatology in June, 2006, volume 43(6), pp. 1231-1240.

2.0 SUMMARY

The liver plays an increasingly recognized role in the host's immune responses. The direct contribution of hepatocytes as effector cells to local immunity, pathogen containment, and liver disease is not determined. The present in vitro study examined whether hepatocytes can eliminate other cells via CD95L/CD95-mediated mechanism and whether this cytotoxic activity can be modulated by cytokines such as IFN γ or TNF α . We have found that normal woodchuck and human hepatocytes, both cultured and primary freshly isolated, as well as human HepG2 cells, intrinsically transcribe not only CD95 but also CD95L when examined by reverse transcription-polymerase chain reaction (RT-PCR) assays. The functional competence of CD95L, which was detectable in hepatocytes and HepG2 cells by Western blotting, was confirmed in bioassays by induction of apoptosis of CD95-bearing P815 and LS102.9 cell targets and validated by inhibition of the cell killing with CD95 antagonistic antibody or with a general caspase inhibitor. Further, exposure of cultured hepatocytes to IFN γ or their stable transfection with IFN γ cDNA or TNF α cDNA increased hepatocyte CD95L/CD95-mediated cell killing. In conclusion, our findings indicate that hepatocytes express both CD95L and CD95 and they can induce death of other cells by the CD95L-dependent mechanism. They also show that IFN γ and, to a lesser extent, TNF α can enhance hepatocyte CD95L-mediated cytotoxicity. This suggests that the

local cytokine environment may modulate hepatocyte contribution to the liver immunity.

2.1 INTRODUCTION

The removal of antigens and aberrant cells from circulation is a physiological function of the liver, making this organ an important contributor to the first line of host immune defense. After birth, the liver is the site of production of immune competent cells, including NK, NKT and extrathymic T cells (Golden-Mason and O'Farrelly, 2002), elimination of activated CTL (Belz et al., 1998; Kennedy et al., 2001; Mehal et al., 1999; Russell et al., 1998), and synthesis of complement and acute phase proteins (Baumann et al., 1993). The liver is considered to be an immunocompetent organ whose contributions towards induction of peripheral immunotolerance and surveillance against pathogens are increasingly recognized. The role of Kupffer cells and sinusoidal endothelial cells in clearance of antigens and cells has been well established (Mehal et al., 2001; Smedsrod et al., 1990). However, whether hepatocytes can directly eliminate cells either passing through, trapped or normally residing in hepatic parenchyma remains unknown. In this regard, the endowment of hepatocytes with ASGPR may not only facilitate removal of desialylated glycoproteins from plasma (Diao et al., 2003; Stockert, 1995), but also provide a contact between hepatocyte and other cells through recognition of their surface proteins depleted of terminal sialic acid residues. This may initiate a cascade of events leading to elimination of the cells contacted by hepatocytes. In the current study, we examined whether hepatocytes are equipped in the molecular

machinery to act as cytotoxic effectors and, if so, whether this activity can be modified by the local cytokine milieu.

The cytotoxic cells can induce cell death through interaction of their membrane-associated CD95L with CD95 receptor on cell targets (Itoh et al., 1991; Suda et al., 1993) or by release of cytolytic proteins, perforin and granzyme B (Kagi et al., 1994). CD95L-induced cell death is a main mechanism by which activated CTL eliminate targeted cells (Roth and Pircher, 2004). In this regard, it has been shown that IFN γ can enhance the CTL CD95L-mediated cell killing (Roth and Pircher, 2004). The CD95L-CD95 interaction also is crucial for removal of autoreactive T cells during thymic maturation (Kishimoto et al., 1998) and in homeostatic protraction of T and B lymphocytes (Brunner et al., 1995; Lam et al., 1997). It is evident that lymphoid cells can display both CD95L and CD95 and act depending upon circumstances either as cytotoxic effectors or as targets for the cytotoxic reactions. Furthermore, expression of CD95L is not restricted to activated T cells, NK and NK T cells (Oshimi et al., 1996; Russell and Ley, 2002), but is also evident in cells at the immune privileged sites (Griffith et al., 1995).

Considering normal liver tissue, hepatocytes were found to be CD95L nonreactive by immunohistochemical methods (Afford et al., 1999; Roskams et al., 2000). However, the ligand expression was detected by immunostainings or in situ hybridization in hepatocytes in HCC (Roskams et al., 2000), alcoholic hepatitis (Galle et al., 1995), liver allograft rejection (Afford et al., 1999), and Wilson's disease (Strand et al., 1998). This may suggest that CD95L is in fact expressed in normal hepatocytes but at levels which are not detectable by conventional methods, while hepatocytes in

some pathological conditions carry the ligand at quantities more readily identifiable by the same approaches.

In contrast to CD95L, it is well established that hepatocytes are CD95-bearing cells and their apoptosis can be swiftly induced by anti-CD95 antibody (Ogasawara et al., 1993) or by binding of CD95L (Nakamoto et al., 1997). Therefore, it has been postulated that certain liver diseases or their distinctive morphological stages could be a consequence of interaction between a hepatocyte's CD95L and its cognate receptor in either a suicidal or fratricidal manner (Afford et al., 1999; Galle et al., 1995; Roskams et al., 2000; Strand et al., 1998).

In the present work, we investigated whether normal cultured hepatocytes, primary hepatocytes and HepG2 cells, manifest a phenotype of cytotoxic effector cells and, if so, whether they can kill other cells via a CD95L-activated pathway. Although the presence and functionality of the cytotoxic machinery was investigated using primary hepatocytes obtained from healthy human liver, in the majority of experiments we used hepatocytes from healthy woodchucks known to be susceptible to WHV. These animals represent an excellent natural model of HBV infection (Michalak, 2000; Tennant et al., 2004). We utilized woodchuck hepatocytes as cytotoxic effectors to facilitate future investigations on their potential involvements in the liver innate immunity and the development of different forms and outcomes of viral hepatitis. In addition, due to the critical role of intrahepatic IFN γ and TNF α in the recovery or progression to chronic viral hepatitis (Hodgson and Michalak, 2001; Wieland et al., 2004a), the effect of these cytokines on the hepatocyte-mediated cell killing was investigated.

2.2 MATERIALS AND METHODS

2.2.1 Cell lines

Woodchuck WCM-260 hepatocyte line was established from the liver of a healthy woodchuck, as reported (Diao et al., 1998; Lew and Michalak, 2001). The cells were maintained in Hepato-STIM culture medium (Becton Dickenson, Bedford, MA) and demonstrated stable growth, consistent morphology, and transcribed albumin and ASGPR when passaged weekly (Diao et al., 1998; Lew and Michalak, 2001). Human HepG2 cells (ATCC HB-8065; American Type Culture Collection, Rockville, MD), and murine mastocytoma P815 (ATCC TIB-64) and lymphoma LS102.9 cells (ATCC HB-97), both constitutively bearing CD95 (Ebata, 2001; Hodgson, 1999), were cultured as reported (Diao et al., 1998; Hodgson et al., 1999; Lew and Michalak, 2001).

2.2.2 Preparation of hepatocytes and lymphoid cells

Hepatocytes were isolated from livers of healthy woodchucks by two-step collagenase microperfusion (Churchill and Michalak, 2004). Preparations were at least 98% pure by phase-contrast microscopy. PBMC were prepared from the same animals and from a healthy human by Ficoll-Hypaque gradient centrifugation (Pharmacia Biotech, Quebec, Canada) (Lew and Michalak, 2001). Primary human hepatocytes were similarly isolated from healthy portions of resected liver tissue and were subsequently cryopreserved and supplied by Dominion Pharmakine (Derio-Bizkaia, Spain). Animal experimental protocols were approved by the Institutional Presidents' Committee on Animal Bioethics and Care.

2.2.3 Treatment of hepatocytes with IFN γ or TNF α

WCM-260 hepatocytes were treated with woodchuck IFN γ (wIFN γ) or TNF α (wTNF α) using two approaches. First, the cells were exposed to bioactive recombinant wIFN γ or wTNF α (rwIFN γ and rwTNF α , respectively) produced in the baculovirus expression system, as recently reported (Wang and Michalak, 2005). After 18-h exposure to 10-fold dilutions of the cytokines, cytotoxic activity of WCM-260 was measured (see below) and compared to naive hepatocytes or those exposed to culture supernatant from insect cells infected with wild-type baculovirus (Wang and Michalak, 2005). In addition, mRNAs from WCM-260 hepatocytes treated with rwIFN γ (150 U/ml) or rwTNF α (35 U/ml) were analyzed (see below). Secondly, WCM-260 were stably transfected with the complete wIFN γ or wTNF α cDNA (GenBank accession number AF232728 for wIFN γ (Michalak et al., 2000) and AF333967 for wTNF α (Hodgson and Michalak, 2001)) using pcDNA3.1 vector-based expression constructs (Invitrogen, Carlsbad, CA) and Lipofectamine 2000 (Invitrogen). Cells transfected with empty pcDNA3.1 were used as controls. Transcriptional activity of wIFN γ and wTNF α in WCM-260 hepatocytes was ascertained by RT-PCR using primers and conditions reported (Wang and Michalak, 2005). To verify biological activity of the cytokines expressed, WCM-260 hepatocytes exposed to or transfected with wIFN γ or wTNF α cDNA were examined for MHC class I antigen display by flow cytometry (Wang and Michalak, 2005).

2.2.4 RNA extraction and reverse transcription

Total RNA was extracted from 2×10^5 - 1×10^6 cultured or primary woodchuck and human hepatocytes, HepG2 cells, woodchuck or human PBMC, and from 100 mg of normal woodchuck spleen using Trizol reagent (Invitrogen). RNA (2 μ g) was reverse transcribed to cDNA in a 20- μ l reaction volume with 200 U Moloney murine leukemia virus reverse transcriptase (Invitrogen), as described before (Coffin and Michalak, 1999).

2.2.5 Cloning of woodchuck gene sequences

For cloning of woodchuck CD95L and CD95, cDNA transcribed from woodchuck spleen or PBMC RNA was amplified by PCR using degenerate primers deduced through interspecies comparison of sequences available in GenBank. PCR amplicons were cloned into the dual promoter vector PCRII using the TOPO TA cloning system (Invitrogen) and sequenced. Woodchuck-specific primers spanning introns were designed, the gene fragments amplified, cloned, their sequence determined and submitted to GenBank (accession numbers AF152368 and AY993960 for woodchuck CD95L and CD95, respectively).

2.2.6 RT-PCR and Southern blot hybridization

Transcription of CD95L and CD95 genes in test cDNA samples was assessed by PCR. Thus, the CD95L 508-base pair (bp) fragment was amplified with sense primer 5'-CAGCTCTTCCACCTGCAGAAGG and antisense primer 5'-AGATTCCTCAAAATTGATCAGAGAGAG during 32 cycles, each cycle at 95 $^{\circ}$ C for 15 sec, 55 $^{\circ}$ C for 30 sec, and 72 $^{\circ}$ C for 60 sec. Detection of the CD95 500-bp fragment

was facilitated with sense primer 5'-GATGGAGGGCATGGTTTAGAAGTG and antisense primer 5'-AGCAGCTGGAGTTTCTGCTCAGC during 34 cycles, each cycle at 95 °C for 15 sec, 60 °C for 30 sec, and 72 °C for 60 sec. Woodchuck β -actin was amplified as a loading control (Hodgson and Michalak, 2001). Specificity of the PCR products was routinely confirmed by Southern blotting with appropriate probes and autoradiography (Hodgson and Michalak, 2001; Michalak et al., 1999).

2.2.7 Real-time RT-PCR

To quantify expression of CD95L, CD95 and β -actin, real-time RT-PCR was established using the Lightcycler Faststart Master SYBR I kit (Roche Diagnostics, Laval, Quebec, Canada) and the Roche LightCycler (Roche Diagnostics). Reactions were performed in 20- μ l volumes, each containing 2 μ l of cDNA derived from approximately 50 ng RNA using the following primer pairs: sense primer 5'-CCATTTAACAGGTAAGCCC and antisense primer 5'-TCATCATCTTGCCCTCC for CD95L, sense primer 5'-GTGCACCACGTGTGAACATGGAAT and antisense primer 5'-TAATCGGGAGTAGCAGTAGCAGGA for CD95, and primers 5'-CAACCGTGAGAAGATGACC and 5'-ATCTCCTGCTCGAAGTCC for β -actin.

2.2.8 CD95L detection by Western blotting

Cell suspensions were treated with ice cold RIPA buffer (1% NP-40, 0.5% DOC, 0.1% SDS, 150 mM NaCl in 50 mM Tris, pH 8.0) and cellular debris removed by centrifugation. Lysates were separated by SDS-PAGE at 10-20 μ g protein/lane and then blotted onto a nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ)

by semi-dry transfer using the Bio-Rad SD cell system (Bio-Rad, Mississauga, Ontario, Canada) (Michalak et al., 2000). As positive controls, freshly isolated human and woodchuck PBMC were examined at 20 µg protein/lane. The blots were blocked with 5% skim milk in Tris-buffered saline, pH 7.4, overnight at 4 °C. Both transmembrane (38 kDa) and soluble (28 kDa) isoforms of CD95L were detected using rabbit anti-murine CD95L IgG (Santa Cruz Biotech, Santa Cruz, CA) followed by HRP-conjugated goat anti-rabbit IgG F(ab')₂ antibodies (Jackson ImmunoResearch, West Grove, PA). The signals were visualized using an ECL detection kit (Sigma, Oakville, Ontario, Canada).

2.2.9 Preparation of target cells for cytotoxicity assays

P815 and LS102.9 cells were subcultured to a density of 5×10^5 cells/ml and labeled with 50 µCi of ³H-adenine (Perkin Elmer, Wellesley, MA) for 18 h prior to the JAM cytotoxicity assay. The cells were washed 3 times in HBSS (Invitrogen), resuspended in RPMI medium³⁴ at 1×10^5 cells/ml, and immediately used.

2.9.10 JAM cytotoxicity (DNA fragmentation) assay

WCM-260 hepatocytes, HepG2 cells or WCM-260 transfected with wINF-γ or wTNF-α cDNA were grown to confluence ($\sim 6 \times 10^4$ cells/well) in 96-well flat-bottom cell culture plates. 293 human embryonic kidney (293HEK; ATCC reference number CRL-1573) cells were utilized as effector cells as a noncytolytic negative control (data not shown). In some experiments, WCM-260 were treated with rwIFN_γ or rwTNF_α at $\sim 95\%$ confluence for 18 h prior to the assay. In the case of primary hepatocytes, the cells were aliquoted at 6×10^4 /well in 200-µl volumes. ³H-adenine-labeled P815 or

LS102.9 cells were added at 20×10^3 , 10×10^3 , 5×10^3 and, occasionally, at 40×10^3 cells in quadruplicate to experimental wells yielding final effector:target (E:T) ratios of 6:1, 3:1, 1.5:1 and 12:1, respectively. Plates were centrifuged for 5 min at $45 \times g$ and incubated at 37°C in 5% CO_2 for 18 h. Well contents were harvested onto glass fibre mats (Perkin Elmer, Wellesley, MA) using a 96-well harvester (Tomtec, Hamden, CT). Counts per minute (cpm) were measured using a Top10 beta counter (Becton Dickinson, San Diego, CA) and percent lysis was determined by applying the formula: percent specific lysis = (control cpm - experimental cpm)/control cpm \times 100, where control cpm for each target cell type were obtained in the absence of effector cells. Freshly isolated woodchuck PBMC were used in parallel experiments at E:T ratios of 50:1, 25:1 and 12.5:1, as described before (Hodgson et al., 1999) (data not shown). Specificity of CD95L-mediated lysis of P815 cells was confirmed using an antagonistic anti-CD95 monoclonal antibody (mAb) (Jo2; Becton Dickinson), as described (Hodgson et al., 1999). In the case of LS102.9 cells, caspase-dependent initiation of apoptosis was blocked by pre-treatment of the cells with the pan-caspase inhibitor z-VAD-fmk (Biomol, Plymouth, PA) at $150 \mu\text{M}$ for 4 h.

2.9.11 Statistical analyses

Results were analyzed by unpaired Student's *t* test with Welch's correction using GraphPad Prism software (GraphPad Software Inc., San Diego, CA). Differences between experimental conditions were considered to be significant when two sided *P* values were less than 0.05.

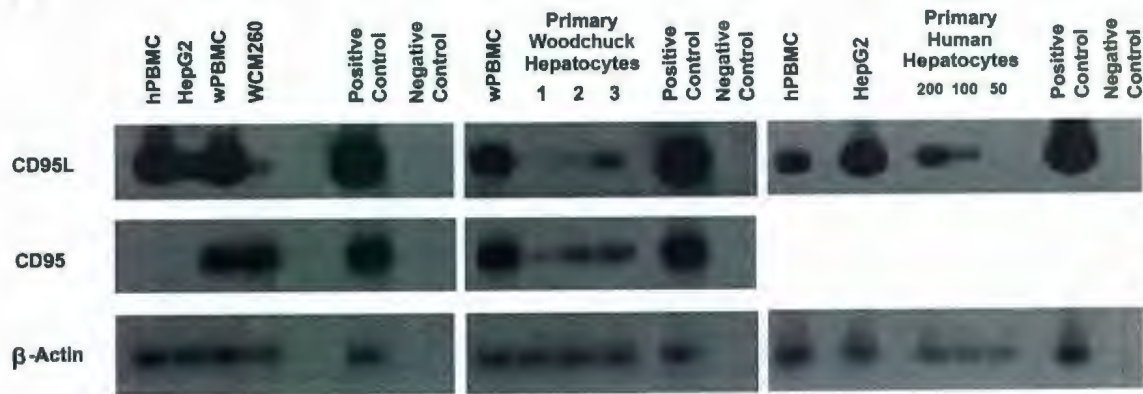
2.3 RESULTS

2.3.1 Hepatocytes constitutively transcribe CD95L

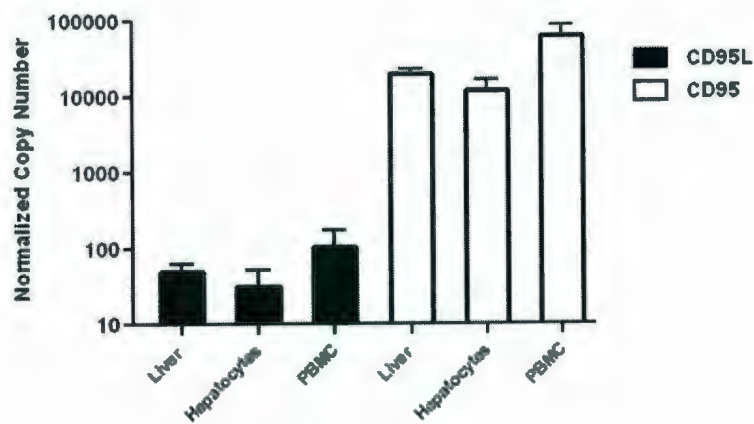
Initial experiments were performed using a non-quantitative RT-PCR to determine whether hepatocytes express genes encoding for the CD95L and its receptor. As shown in Figure 2.1A, CD95L mRNA was without difficulty detected in human and woodchuck lymphoid cells, as well as in HepG2 cells and in woodchuck WCM-260 hepatocytes. Importantly, pure preparations of isolated woodchuck and human hepatocytes also displayed CD95L mRNA. In addition, transcription of CD95 mRNA was evident in woodchuck PBMC and hepatocytes, although CD95 mRNA was not detected in human PBMC and HepG2 cells due to the woodchuck-restricted specificity of the CD95 PCR primers used (Fig. 2.1A).

When expression of CD95L was quantified by real-time RT-PCR in samples of whole liver tissue, in hepatocytes isolated from these livers, and in autologous PBMC, no statistically significant differences in the mean levels of CD95L mRNA were found (Fig. 2.1B). Similarly, the mean CD95 mRNA levels were not statistically different between the liver, primary hepatocytes and peripheral lymphoid cells (Fig. 2.1B). A possibility that primary hepatocytes might be contaminated with lymphoid cells was

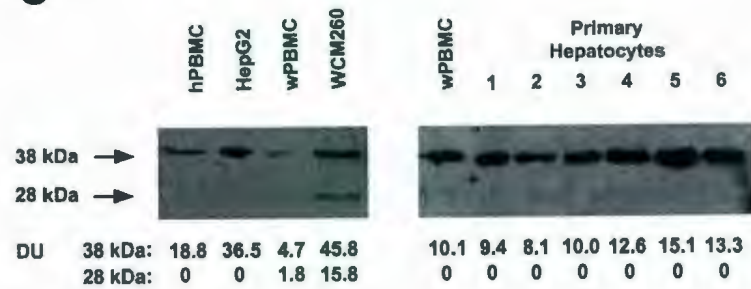
A



B



C



excluded based on the absence of CD3 cDNA signals (data not shown), as analyzed by RT-PCR using conditions previously reported (Hodgson and Michalak, 2001).

2.3.2 Hepatocytes display CD95L protein

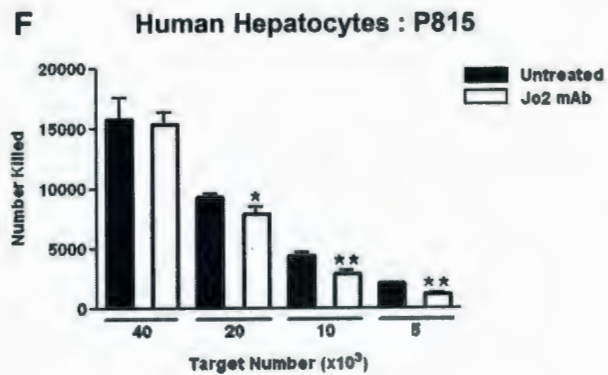
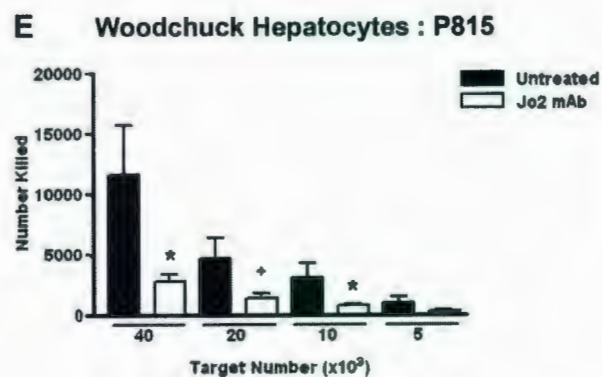
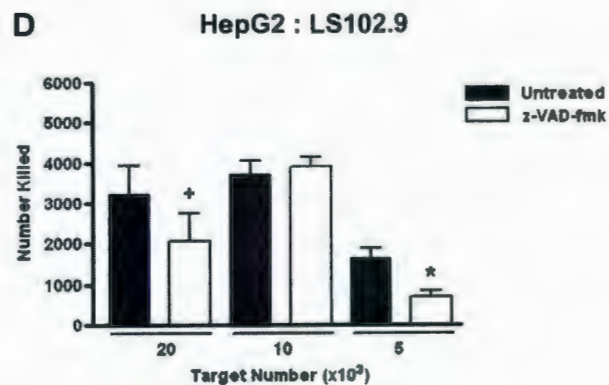
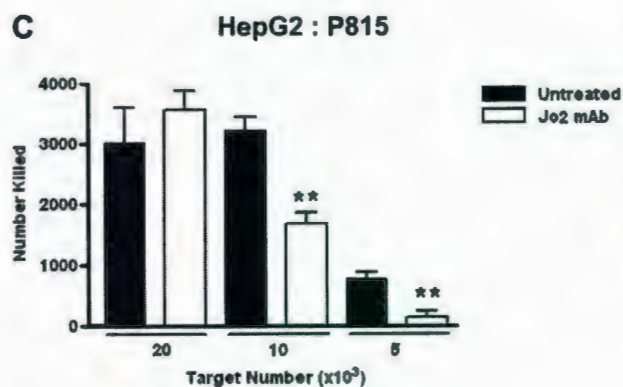
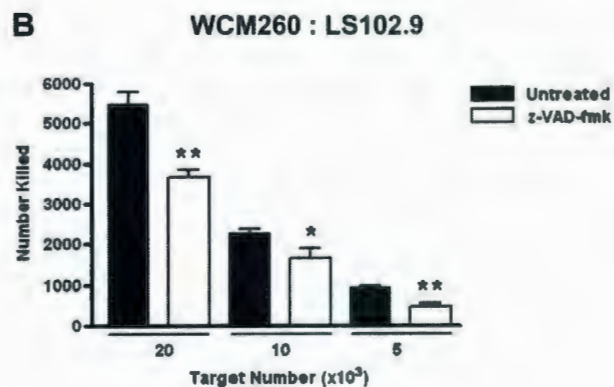
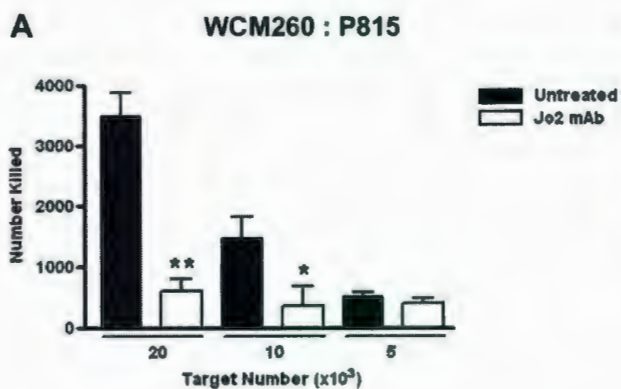
To determine whether CD95L mRNA transcription was accompanied by CD95L protein, hepatocyte lysates were probed with anti-CD95L antibody by Western blotting. Fig. 2.1C shows that WCM-260 hepatocytes and HepG2 cells, as well as hepatocytes isolated from 6 different woodchucks, revealed the 38-kDa membrane-bound isoform of CD95L, which was also evident in control woodchuck and human PBMC. In addition, WCM-260 exhibited the 28-kDa isoform representing pre-formed soluble CD95L, which was also detected in woodchuck PBMC (Fig. 2.1C).

Densitometry analysis of the 38-kDa bands suggested that the protein occurred in WCM-260 and HepG2 cells at about 9-fold and 2-fold greater levels than in woodchuck and human PBMC, respectively. Primary hepatocytes contained approximately twice the protein of PBMC isolated from the same woodchucks when adjusted to the same total protein concentration (Fig. 2.1C and data not shown).

2.3.3 Hepatocyte CD95L-mediated cytotoxicity

Hepatocyte cytotoxicity was evaluated using a JAM DNA fragmentation assay which employed constant numbers of hepatocytes as effectors, and increasing numbers of CD95-bearing P815 or LS102.9 cells as targets. As shown in Figures 2.2A and 2.2B, WCM-260 hepatocytes killed both cell types in a manner dependent on the target numbers. The killing was significantly reduced ($P < 0.05$ or $P < 0.001$) after pretreatment of P815 cells with an antagonistic CD95-blocking antibody, Jo2 (Fig. 2.2A), or when LS102.9 cell death was measured in the presence of z-VAD-fmk (Fig. 2.2B). HepG2 cells also killed P815 and LS102.9 targets, although a statistically significant inhibition by Jo2 mAb or z-VAD-fmk was usually seen at lower numbers of

Fig 2.2. Hepatocyte killing of P815 and L102.9 cell targets mediated by CD95L. WCM-260 hepatocytes (A and B), HepG2 cells (C and D) and primary woodchuck (E) or human (F) hepatocytes were used as effectors against CD95-bearing P815 (A, C, E and F) or LS102.9 (B and D) cells in the JAM DNA fragmentation assay. Jo2 mAb was used to block CD95L-dependent killing of P815 cells (A, C, E and F), while lysis of LS102.9 cells was inhibited with pancaspase z-VAD-fmk inhibitor (B and D). Data bars shown in A to D are mean values \pm SEM from three separate experiments with 3-6 experimental wells per condition tested. For E, hepatocytes isolated from 3 healthy woodchucks were used as effector cells, while F demonstrates target cell killing mediated by primary human hepatocytes. Results are shown as mean values \pm SEM, with each experiment having been performed with 4 - 8 experimental wells per each E:T ratio. Data bars marked with ** are significant at $P < 0.005$ and with * at $P < 0.05$ when compared with the cells not treated with Jo2 mAb or z-VAD-fmk inhibitor. P value for data bars marked with + is 0.07.



targets (Figs. 2.2C and 2.2D). Primary hepatocytes isolated from healthy woodchucks (Fig. 2.2E) and those derived from healthy human liver (Fig. 2.2F) also eliminated CD95-sensitive P815 cells. Inhibition of the CD95 ligand-receptor interaction by Jo2 mAb markedly ($P = 0.07$) or significantly ($P < 0.005$ and $P < 0.05$) reduced the level of P815 cell killing.

2.3.4 IFN γ and TNF α upregulate CD95L transcription in hepatocytes

In the first step, to learn whether exposure to IFN γ or TNF α would induce a measurable biological effect in hepatocytes, WCM-260 cells were examined for expression of class I MHC after treatment with rwIFN γ (150 U/ml) or rwTNF α (35 U/ml) or after transfection with the cytokine cDNA. Thus, class I MHC display was increased by 14.2-fold in hepatocytes exposed to rwIFN γ (Fig. 2.3A) and by 12.9.-fold in those transfected with wIFN γ cDNA (Fig. 2.3B). Exogenous rwTNF α increased the expression by 2.4-fold (Fig. 2.3A), whereas transfection wTNF α cDNA induced a 2.8-fold increase (Fig. 2.3B). Therefore, both treatment approaches with IFN γ or TNF α enhanced expression of a functionally important molecule on hepatocytes.

The effect of rwIFN γ or rwTNF α on CD95L mRNA in hepatocytes was quantified by real-time RT-PCR. CD95L mRNA was upregulated ($P < 0.001$) by rwIFN γ , but not by rwTNF α (Fig. 2.3C). CD95L mRNA was also meaningfully ($P < 0.001$) elevated in hepatocytes transfected with wIFN γ or wTNF α cDNA, when compared to controls transfected with empty vector (Fig. 2.3D). These data clearly showed that IFN γ is a potent inducer of CD95L transcription in hepatocytes and suggested that TNF α may exert a similar effect under certain conditions.

2.3.5 IFN γ but not TNF α enhances hepatocyte CD95L-mediated cytotoxicity

As shown in Figure 2.4, exposure of WCM-260 to increasing concentrations of rwIFN γ enhanced hepatocyte cytotoxicity towards P815 and LS102.9 targets. Thus,

Fig 2.3. Evaluation of the effects of IFN- γ and TNF- α on class I MHC antigen display and CD95L gene expression in hepatocytes. The class I MHC heavy chain detection by flow cytometry in WCM-260 hepatocytes (A) exposed to or (B) stably transfected with wIFN- γ or wTNF- α and in control cells. For A, the cells were exposed to 150 U/ml of rwIFN- γ , 35 U/ml of rwTNF- α or to a supernatant from insect cells infected with wild-type baculoviral vector (wt super) for 18 h prior to staining with B1b.B9 mAb. Intact WCM-260 cells not exposed to any of the above and stained with B1b.B9 mAb were used as an additional control. For B, WCM-260 hepatocytes were transfected with wIFN- γ (pcDNA3.1wIFN- γ), wTNF- α (pcDNA3.1wTNF- α) or with empty cDNA3.1 vector and probed with B1b.B9 mAb. Non-transfected WCM-260 were used as an additional control. For C and D, CD95L mRNA was quantified by real time RT-PCR in WCM-260 hepatocytes exposed to rwIFN- γ or rwTNF- α or transfected with these cytokine genes, respectively. Data are represented as mean percentage expression values \pm SEM from 3 separate experiments after normalization to woodchuck β -actin and by taking expression of a given gene in unstimulated hepatocytes (C) or in hepatocytes transfected with empty cDNA3.1 vector (D) as 100%. Data bars marked with ** are significant at $P < 0.001$ relative to respective controls.

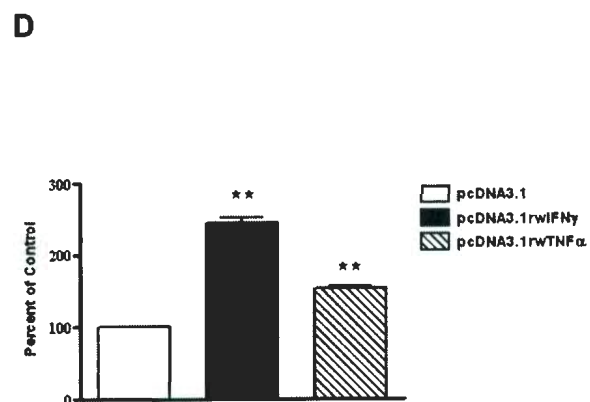
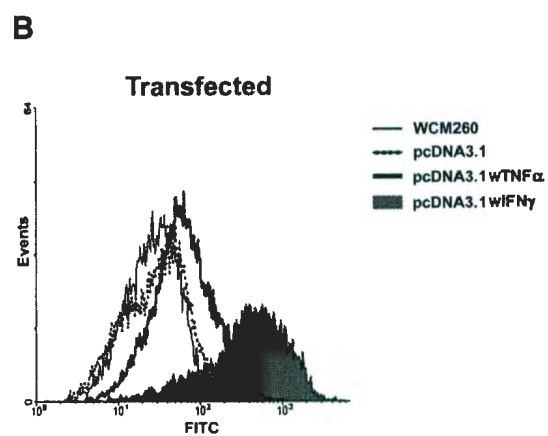
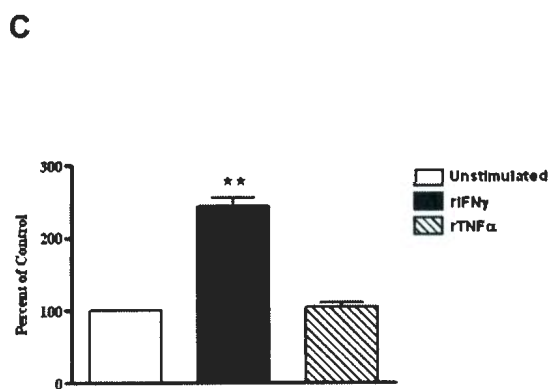
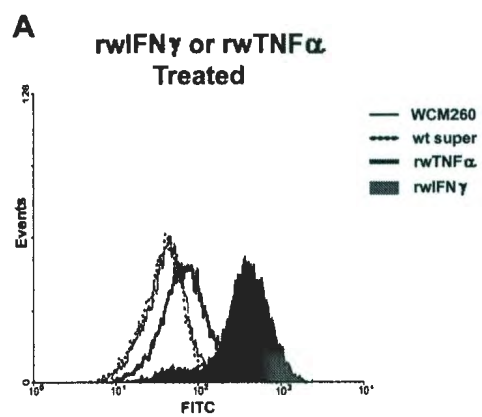
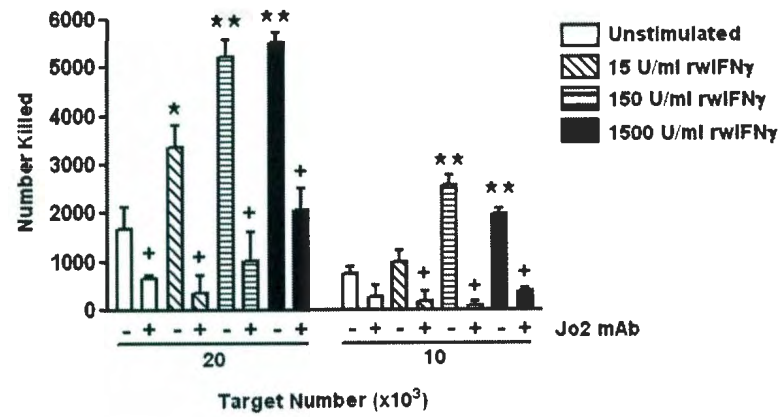


Fig 2.4. Exposure to IFN- γ enhances hepatocyte CD95L-mediated cell killing. WCM-260 hepatocytes were stimulated with 15, 150 or 1500 U/ml rwIFN- γ and used as effectors at 6×10^4 cells/assay against indicated numbers of P815 (A) or LS102.9 (B) targets and their cytotoxicity measured by JAM assay. Data represent mean values \pm SEM obtained from two separate experiments, each performed in duplicate. Data bars marked with ** are significant at $P < 0.01$ and those with * at $P < 0.05$ when compared with cells not exposed to the cytokine (unstimulated). Preincubation with Jo2 mAb reduced killing of P815 cells. Data bars marked with [†] are significant at $P < 0.05$ when compared to target cells not treated with the antibody.

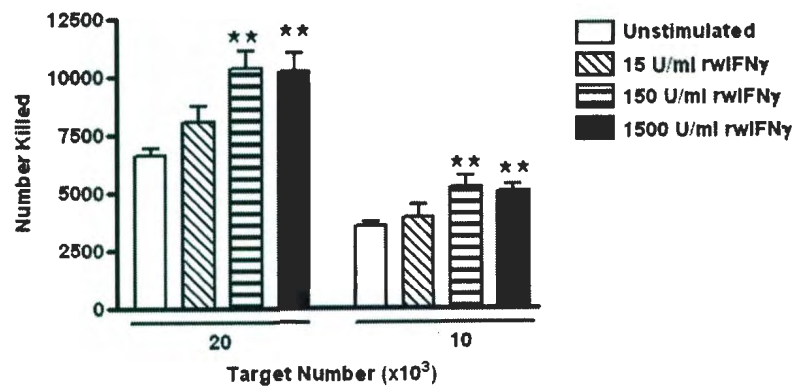
A

WCM260 : P815



B

WCM260 : LS102.9



rwIFN γ significantly ($P < 0.001$) increased hepatocyte killing of P815 (Fig. 2.4A) and LS102.9 (Fig. 2.4B) under the majority of the conditions tested, while rwTNF α had no measurable effect (data not shown). It is important to indicate that an MTT assay showed that neither rwIFN γ nor rwTNF α affected the viability of hepatocytes, P815 or LS102.9 cells at the concentrations used (data not shown), excluding the possibility that the cytokines may affect cell survival during the assay. Furthermore, P815 cell lysis was inhibited by pretreatment with Jo2 mAb (Fig. 2.4A), which was consistent with previous observations (see Figure 2.2).

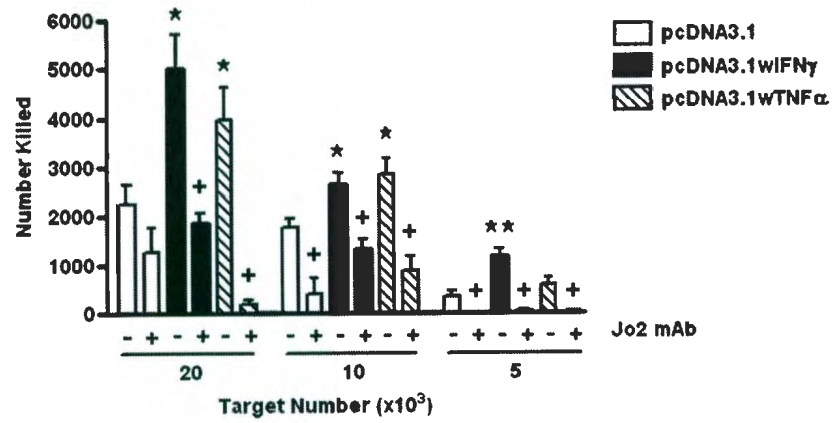
2.3.6 Transfection with IFN γ or TNF α cDNA enhances hepatocyte CD95L-mediated cell killing

As illustrated in Figure 2.5, transfection of WCM-260 hepatocytes with cDNA of either wIFN γ or wTNF α enhanced CD95L-mediated killing of P815 and LS102.9 targets. Thus, transfection with wIFN γ cDNA significantly ($P < 0.01$) increased elimination of P815 cells, which was consistent with the CD95L mRNA upregulation illustrated in Fig. 2.3C.

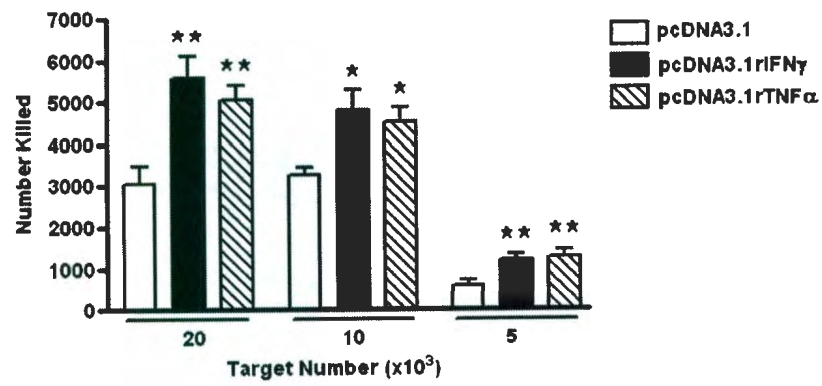
This killing was blocked by 59-85% ($P < 0.05$) after pretreatment of P815 cells with Jo2 mAb (Fig. 2.5A). Elimination of LS102.9 targets was also significantly enhanced ($P < 0.05$ or $P < 0.01$) when compared to control cells transfected with the empty cDNA3.1 vector. Also, transfection of WCM-260 hepatocytes with wTNF α cDNA increased ($P < 0.05$ or $P < 0.01$) their cytotoxic activity towards P815 and LS102.9 targets (Fig. 2.5A and Fig. 2.5B, respectively), which was consistent with increased CD95L mRNA levels seen in Fig. 2.3D.

Fig 2.5. Transfection with IFN- γ or TNF- α cDNA upregulates hepatocyte CD95L-mediated cytotoxicity. WCM-260 hepatocytes stably transfected with wIFN- γ (pcDNA3.1wIFN γ) or wTNF- α (pcDNA3.1wTNF α) were used as effectors at 6×10^4 cells/assay against indicated numbers of P815 (A) or LS102.9 (B) target cells in the JAM assay. Preincubation of P815 cells with Jo2 mAb significantly decreased their killing by hepatocytes. Data bars marked with ** are significant at $P < 0.01$ and with * at $P < 0.05$ when compared with WCM-260 transfected with empty cDNA3.1 vector (pcDNA3.1). Data bars marked with + are significant at $P < 0.05$ when compared to P815 cells not treated with Jo2 mAb.

A WCM260 : P815



B WCM260 : LS102.9



2.4 DISCUSSION

Hepatocytes in normal livers have been considered to be CD95L nonreactive and their ability to induce cell death had not been investigated. In this study, we documented by standard RT-PCR that both cultured hepatocytes and primary hepatocytes from livers not compromised by a disease process constitutively transcribe genes encoding molecules of the CD95L/CD95 pathway. To assess whether CD95L was displayed at functionally adequate levels in hepatocytes, previously established cytotoxic assays with heterologous cells as targets were adopted. The results showed that hepatocytes not only transcribe CD95L mRNA and synthesize the ligand, but can efficiently eliminate other cells via a CD95L-mediated mechanism. Furthermore, the CD95L-dependent cell killing was enhanced when hepatocytes were exposed to IFN- γ or transfected with IFN- γ or TNF- α cDNA, suggesting that hepatocyte cytotoxicity *in vivo* can be modulated by these cytokines.

Expression of the genes encoding effector molecules of the CD95L/CD95 pathway was until now an attribute of activated T cells, NK and NK T cells (Oshimi et al., 1996; Russell and Ley, 2002). However, our analysis showed that the levels of CD95L and CD95 transcription in hepatocytes and lymphoid cells were not meaningfully different (Fig 2.1). However, both cultured and freshly isolated hepatocytes displayed greater amounts of CD95L protein than lymphoid cells, suggesting that CD95L cDNA could be translated more efficiently in hepatocytes.

Previous attempts to identify CD95L protein in normal hepatocytes were not successful, however, the ligand has been detected in hepatocytes in some liver disorders and during allograft rejection (Afford et al., 1999; Galle et al., 1995;

Roskams et al., 2000; Strand et al., 1998), and in HepG2 cells (Strand et al., 1998). In our study, a membrane-bound CD95L 38-kDa protein was evident in woodchuck cultured and primary hepatocytes, and in HepG2 cells. In addition, a 28-kDa protein representing the soluble form of the ligand was seen in woodchuck WCM-260 hepatocytes and PBMC. Our success in identifying CD95L could be due to immediate isolation and preservation of RNA and proteins, using detection assays of superior sensitivity, and a remote possibility that woodchuck hepatocytes may carry greater amounts of CD95L transcripts and protein than hepatocytes in the species previously investigated.

Hepatocyte ability to eliminate cell targets via the CD95L/CD95 pathway was examined by the JAM cytotoxicity assay (Ayres et al., 2003; Matzinger, 1991). The validity of the finding that woodchuck and human hepatocytes, as well as HepG2 cells, eliminated CD95-bearing cells was ascertained using P815 pre-incubated with Jo2 mAb. This antibody is uniquely noncytolytic to P815 cells, and blocks recognition of cell surface CD95 by CD95L (Hodgson et al., 1999; Kuwano and Arai, 1996). Additional evidence was provided using LS102.9 cells treated with z-VAD-fmk, an inhibitor of caspase-dependent initiation of apoptosis (Sarin et al., 1998). Thus, utilization of two different cell targets and two inhibitors blocking either CD95 or the intracellular cascade initiated by the CD95 ligand-receptor interaction clearly demonstrated ability of hepatocytes to induce cell death via the CD95L-CD95 mechanism. This hepatocyte property, which is apparent in both healthy woodchuck and human primary hepatocytes, has not been documented before.

Constitutive expression of both CD95L and CD95 in hepatocytes could be considered to be a paradox, however, this finding follows the blueprint seen in different lymphoid cell subtypes in which both molecules are displayed (Suzuki and Fink, 2000). Although at this stage we cannot dissect whether a single hepatocyte expresses both of the molecules simultaneously, it is possible that the display of functional CD95L depends, like in lymphoid cells, on cell activation (Roth and Pircher, 2004; Suzuki and Fink, 2000). Since CD95L protein in lymphoid cells is sequestered intracellularly and directed into secretory vesicles (Kojima et al., 2002), this may prevent or postpone the ligand exposure on the effector surface and initiation of cell death via CD95L-CD95 interaction. The same could be true for hepatocytes.

Because of our interest in the mechanisms of liver injury in viral hepatitis and the fact that early and strong intrahepatic IFN γ and TNF α response is paramount to the resolution of hepadnaviral infection (Hodgson and Michalak, 2001; Wieland et al., 2004a), we wanted to determine whether IFN γ and TNF α may influence hepatocyte CD95L-mediated cytotoxicity. As we uncovered, wIFN γ and, under certain conditions, wTNF α , augment hepatocyte CD95L-dependent cell killing. Since IFN γ has been known to enhance CD95L expression in both lymphoid and nonlymphoid cells (Moers et al., 1999; Roth and Pircher, 2004), the increases in hepatocyte CD95L mRNA and CD95L-dependent cell killing upon exposure to wIFN γ were consistent with the predicted outcomes. In addition, these data provided an independent confirmation that CD95L synthesized by hepatocytes is functionally competent and its expression can be regulated by extracellular factors. Further, CD95L expression can also be enhanced by TNF- α through activation of NF- κ B (Gaur and Aggarwal, 2003), which subsequently

binds to the CD95L promoter (Matsui et al., 1998). Although CD95L mRNA upregulation and an increase in CD95L-dependent cell killing were observed after transfection of hepatocytes with TNF α cDNA, exogenous TNF α did not produce such an effect. The reason behind this is not clear. The data on class I MHC expression suggested that TNF α was recognized and activated downstream signalling in hepatocytes under both treatment conditions. Nonetheless, considering the results as a whole, IFN γ was consistently more potent than TNF α in enhancing hepatocyte CD95L-mediated cell killing.

The identification in this study of the constitutive expression of functionally competent CD95L in hepatocytes and demonstration that IFN γ and, to some degree, TNF α can modulate hepatocyte CD95L-dependent cell killing provide new insights into the intrinsic properties of liver parenchyma. Our results imply that hepatocytes are not just passive objects of actions exerted by other cells, but they can be active players determining fate of the cells brought in contact with their surface. Their cytotoxic activity may contribute to physiological and post-hepatectomic regeneration of liver parenchyma (Desbarats and Newell, 2000), removal of aberrant hepatocytes (Galle et al., 1995; Strand et al., 1998), and elimination of activated T lymphocytes (Kennedy et al., 2001; Mehal et al., 2001), including those specific for viral pathogens. It also is conceivable that hepatocyte cytotoxic activity, which is not readily apparent in normal liver, but can be heightened during inflammatory processes, may contribute to the pathogenesis of different forms of a given liver disease, including viral hepatitis which is normally accompanied by upregulated intrahepatic expression of IFN γ and TNF α (Hodgson and Michalak, 2001). These issues will require further examination.

Overall, the results of our study imply that investigations on immunological processes engaging the liver need to consider hepatocytes as active contributors.

CHAPTER THREE: HEPATOCYTES CAN INDUCE DEATH OF CONTACTED CELLS VIA PERFORIN-DEPENDENT MECHANISM

This study has been accepted for publication in Hepatology, January 2008 (In press)

3.0 SUMMARY

The liver displays unique immunological properties including the ability to remove aberrant cells and pathogens and to induce peripheral immunotolerance. We have previously demonstrated that hepatocytes can cause cell death by a CD95L-mediated mechanism. Here, we provide evidence that hepatocytes can kill other cells via a perforin-dependent pathway. Using cultured woodchuck hepatocytes and human liver cells, as well as freshly isolated woodchuck, mouse and human hepatocytes, we show that hepatocyte-mediated death of CD95-deficient target cells requires microtubule polymerization, a feature of the granule exocytosis-mediated cytotoxicity. Neutralizing anti-perforin antibodies and short hairpin RNA (shRNA) directed against perforin mRNA confirmed the involvement of perforin in hepatocyte-mediated cell killing. In conclusion, this study shows that hepatocytes express biologically competent perforin capable of killing susceptible cells and emphasizes the role of hepatocytes as cytotoxic effectors. This also is the first demonstration of perforin in a non-lymphoid cell type.

3.1 INTRODUCTION

It is now acknowledged that the liver is an immunocompetent organ capable of performing multiple immunological functions, including removal of aberrant cells, containment of pathogens and maintenance of peripheral immune tolerance (Crispe,

2003; Racanelli and Rehermann, 2006). The liver also plays a central role in elimination of activated T lymphocytes. In this regard, the intrahepatic accumulation of activated T cells has been observed after their systemic activation with anti-CD3 antibodies, in extrahepatic viral infections, and after antigen-specific stimulation of transgenic T cell receptors (Crispe, 2003). Although a mechanism underlying T cell retention is unknown, it is presumed that sinusoidal endothelial cells and macrophages are involved in this process (Park et al., 2002). However, the structure of hepatic vasculature also allows for direct contact between lymphocytes and hepatocytes which underlie the sinusoidal lining (Warren et al., 2006). It is therefore feasible that hepatocytes also recognize and act on activated T cells, as well as on other circulating cells. This hepatocyte-lymphocyte interaction could be facilitated by the binding of the B220 (CD45RO) epitope, which is displayed on activated lymphocytes (Huang et al., 1994), to the essentially hepatocyte-restricted ASGPR (Ashwell and Harford, 1982).

We have reported that normal primary and cultured hepatocytes constitutively express CD95L and are capable of inducing death of CD95-bearing cells (Guy et al., 2006). Cytokines such as IFN γ and TNF α , enhance expression of CD95L in hepatocytes and augment CD95L-dependent cell killing *in vitro* (Guy et al., 2006). In addition, expression of functional CD95L can be induced on hepatocytes by transfection with HCV core protein (Ruggieri et al., 2003) and there is an increased apoptosis of intrahepatic T cells in HCV transgenic mice (Iken et al., 2006). Taken together, the cytotoxicity of hepatocytes can be modified by a viral infection and/or an inflammatory process engaging the liver. Nevertheless, a blockage of either CD95 on cells targeted by hepatocytes (Guy et al., 2006) or CD95L on hepatocytes (Iken et al.,

2006) does not completely abrogate hepatocyte-mediated cytolysis, implying that hepatocytes may also cause cell death by a CD95L-independent mechanism.

The granule exocytosis pathway has been recognized as a key element controlling the initial expansion of CD8⁺ CTL and the homeostatic contraction of activated CTL (de Saint Basile and Fischer, 2003), and is required for efficient lysis of virally infected or malignant cells (Trapani and Smyth, 2002). Its function is attributed to polarized release of the cytolytic protein perforin and associated serine proteases which culminate in perforin-mediated membrane remodeling, mannose-6-phosphate receptor-mediated uptake of granzymes, and perforin-facilitated disruption of endosomal trafficking (Trapani and Smyth, 2002).

Because recent findings indicate that hepatocytes can induce cell death and that the CD95L-dependent mechanism may not be the only one involved in this process (Guy et al., 2006; Iken et al., 2006), we examined whether hepatocytes express perforin and can utilize the granule exocytosis pathway to kill other cells. Although we investigated primary mouse and human hepatocytes, the majority of the experiments were performed using hepatocytes from woodchucks known to be susceptible to WHV. The woodchuck-WHV system is an excellent natural model of human HBV infection in which the contribution of hepatocytes to liver immunity and disease can be investigated (Hodgson and Michalak, 2001; Michalak, 2000). Demonstration that hepatocytes can initiate cell death by both CD95L- and perforin-dependent mechanisms and that these activities can be differentially modulated by the cytokine milieu further support the notion that hepatocytes may play diverse effector roles in both physiological and immunopathological processes occurring in the liver.

3.2 MATERIALS AND MEHTODS

3.2.1 Cell lines

Human liver HepG2 cells (ATCC HB-8065; American Type Culture Collection, Rockville, MD), CD95-bearing murine mastocytoma P815 cells (ATCC TIB-64), and CD95-deficient human chronic myelogenous leukemic K562 cells (ATCC CCL-243) were cultured as described (Guy et al., 2006; Hodgson et al., 1999). Jurkat cells (ATCC TIB-152) and CTLL-2 cells (ATCC TIB-214) were maintained according to ATCC guidelines. Woodchuck WCM-260 hepatocyte cell line was established from the liver of a healthy woodchuck and were maintained as reported (Churchill and Michalak, 2004; Diao et al., 1998).

3.2.2 Primary hepatocytes and lymphoid cells

Hepatocytes were isolated from livers of outbred CDI mice (Charles River Laboratories, Wilmington, MA), rats and healthy woodchucks by two-step collagenase microperfusion (Churchill and Michalak, 2004). Preparations were at least 98% pure by phase-contrast microscopy. T and NKT cell contamination was excluded by RT-PCR for CD3 mRNA (Guy et al., 2006; Hodgson and Michalak, 2001). Presence of NK and NKT cells was excluded by negative RT-PCR for NKp46 mRNA using sense primer 5'-TTGCCACCTAGTGACAG and antisense primer 5'-CACCAGGAGCATCACC. PBMC were prepared by Ficoll-Hypaque gradient centrifugation (Pharmacia Biotech, Quebec, Canada) (Hodgson et al., 1999). Pure primary human hepatocytes (DPK-HCWP-H) isolated by microperfusion of a healthy portion of the liver of a 58-year old male donor were supplied by Dominion

Pharmakine (Derio-Bizkaia, Spain). Animal experimental protocols were approved by the Institutional Presidents' Committee on Animal Bioethics and Care.

3.2.3 Hepatocyte treatments with recombinant IFN γ or TNF α

WCM260 hepatocytes were exposed to rwIFN γ or rwTNF α generated in the baculovirus expression system (Wang and Michalak, 2005). Cells were treated for 18 h with 150 U/ml of rwIFN γ or 35 U/ml of rwTNF α or were left untreated.

3.2.4 RNA extraction and reverse transcription

Total RNA was extracted from cultured or primary hepatocytes, HepG2 cells and PBMC using Trizol reagent (Invitrogen, Carlsbad, CA). DNA contamination was removed using a DNase digestion kit (Sigma-Aldrich, Oakville, Ontario, Canada). RNA (2 μ g) was reverse transcribed to cDNA as described (Guy et al., 2006).

3.2.5 Cloning of woodchuck gene sequences

Cloning of woodchuck perforin, granzyme B and NKp46 gene fragments was performed by RT-PCR utilizing degenerate oligonucleotide primers using a strategy as previously described (Hodgson and Michalak, 2001). Woodchuck perforin, granzyme B and NKp46 sequences were submitted to the GenBank under accession numbers AF298158, AY993961 and EF526216, respectively.

3.2.6 RT-PCR and Southern blot analysis

A 302-bp fragment of perforin sequence was amplified with sense primer 5'-GCATCAACAATGACTGGCGGG and antisense primer 5'-TGAAGTGGGTGCCGTAGTTGTGG. The granzyme B 204-bp fragment was detected using sense primer 5'-CCCAGACTATGATGCT and antisense primer 5'-CAACTTTACCTCCTGC. Woodchuck β -actin cDNA was amplified in parallel as a loading control (Guy et al., 2006). Specificity of the PCR products was confirmed by Southern blot hybridization (Hodgson and Michalak, 2001).

3.2.7 Real-time RT-PCR

Real time RT-PCR was established using the LightCycler Faststart Master SYBR I kit (Roche Diagnostics, Laval, Quebec, Canada) and the Roche LightCycler (Roche Diagnostics) using the perforin-specific primer pair outlined above and β -actin-specific primers reported before (Guy et al., 2006).

3.2.8 Perforin detection by Western blotting

Cell suspensions were treated at 4 °C overnight with lysis buffer containing 10% glycerol, 1% Nonidet P-40, 200 mM sodium vanadate, 200 mM sodium fluoride and a protease inhibitor cocktail (Sigma-Aldrich). Proteins were separated at 60 μ g per lane by PAGE under non-reducing conditions and transferred to Immobilon-P membrane (Millipore, Cambridge, Ontario, Canada). Lysates of rat spleen and mouse brain served as positive and negative controls, respectively. Blots were incubated with rabbit anti-perforin antibodies obtained from two different sources (Torrey Pines BioLabs,

Houston, TX and Cell Signaling Technology, Danvers, MA) at 10 µg protein/ml for 18 h at 4 °C. A final incubation with HRP-conjugated secondary antibody for 1 h at ambient temperature was followed by visualization using an ECL detection kit (Perkin Elmer, Wellesley, MA).

3.2.9 Perforin detection by flow cytometry

Suspensions containing 1×10^6 WCM-260 hepatocytes, HepG2 cells, primary mouse hepatocytes or mouse CTLL-2 cells, as perforin-positive controls, were fixed in 2% paraformaldehyde for 20 min, permeabilized with 0.1% Triton-X in the presence of 10% normal goat serum, and incubated with anti-perforin antibodies (Cell Signaling Technology) at 1:100 dilution or rabbit IgG overnight at 4 °C. Cells were washed 3 times by centrifugation, stained with Cy-5-goat anti-rabbit antibodies (Jackson ImmunoResearch, West Grove, PA), and analyzed using a BD FacsCalibur flow cytometer (BD Biosciences, Mississauga, ON, Canada).

3.2.10 JAM cytotoxicity (DNA fragmentation) assay

WCM-260 hepatocytes and HepG2 cells or WCM-260 treated with rwIFN γ or rwTNF α were grown to confluence ($\sim 6 \times 10^4$ cells/well) in 96-well flat-bottom cell culture plates (Guy et al., 2006). Primary fresh mouse, woodchuck or human hepatocytes were aliquoted at 6×10^4 /well in 200-µl volumes. ^3H -thymidine-labeled P815 or K562 cells were added at 20×10^3 , 10×10^3 , 5×10^3 and, occasionally, at 40×10^3 cells in 4 -8 experimental wells yielding final effector : target (E:T) ratios of 3:1, 6:1, 12:1 and 1.5:1, respectively. Plates were centrifuged for 5 min at 45 x g and

incubated at 37 °C in a 5% CO₂ atmosphere for 18 h. Well contents were harvested onto glass fibre mats (Perkin Elmer) using a 96-well harvester (Tomtec, Hamden, CT). Counts per minute (cpm) were measured using a Top10 beta counter (Becton Dickinson) and percent lysis was determined as previously reported (Guy et al., 2006). Freshly isolated woodchuck and human PBMC were used in parallel as controls (Guy et al., 2006). Specificity of CD95L-mediated lysis of P815 cells was confirmed using an antagonistic anti-CD95 Jo2 mAb (Becton Dickinson), as reported (Guy et al., 2006; Hodgson et al., 1999). Inhibition of microtubule-dependent granule release was facilitated using 1 mM colchicine (Sigma-Aldrich) (Hodgson et al., 1999), which we determined to be without toxic effect on target cells by MTT survival assay analyses. JAM assays were additionally performed in the presence of neutralizing anti-perforin antibodies (Torry Pines Biolabs) or a control rabbit IgG (Sigma-Aldrich) at final concentrations of 10 µg/ml.

3.2.11 Silencing of perforin gene by RNA interference

Silencing of perforin gene expression was facilitated using PCR generated Pol III promoter-shRNA gene constructs, as recently described (Castanotto et al., 2002). This strategy employed a universal forward primer, denoted as 5'-U6 primer, which was used together with the appropriate reverse primer which targeted either a perforin-specific 19-nt sequence (Ma et al., 2004) or a 19-nt fragment of a control scrambled RNA sequence (Qiagen, Mississauga, ON) (see Fig. 5A and Table Table 1). The resulting 313-bp amplicons were purified using Wizard PCR prep columns (Promega, Madison, WI). Confluent cultures of WCM-260, HepG2 and Jurkat cells, as well as

primary mouse hepatocytes were transfected with 250 ng of the purified products using Lipofectamine 2000 reagent (Invitrogen). After 24 h, cells were collected and evaluated for perforin mRNA by RT-PCR. Cells were examined by an MTT assay to confirm that treatment with either perforin-specific or scrambled shRNA did not impair their viability. In parallel, transfected WCM-260 and HepG2 cells, and primary mouse hepatocytes were seeded in 6-well (for RNA and protein extraction) and 96-well (for JAM assays) tissue culture plates and cultured for 72 h. The level of perforin mRNA was evaluated by RT-PCR and that of perforin protein by Western blotting with anti-perforin antibodies (Cell Signaling Technology), while killing of P815 and/or K562 cells was measured by JAM assay at E:T ratio 1.5:1, as described above.

3.2.12 Statistical analyses

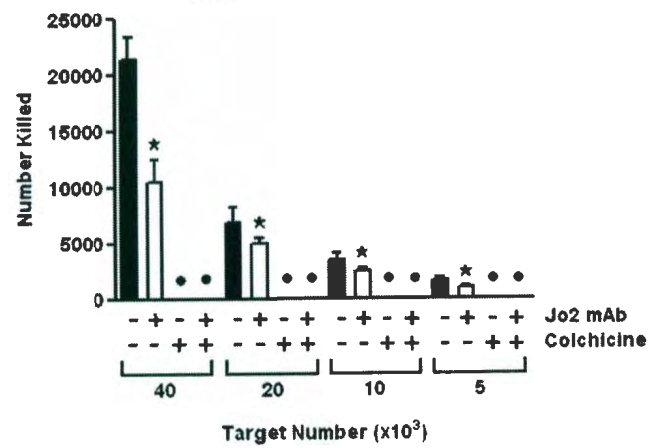
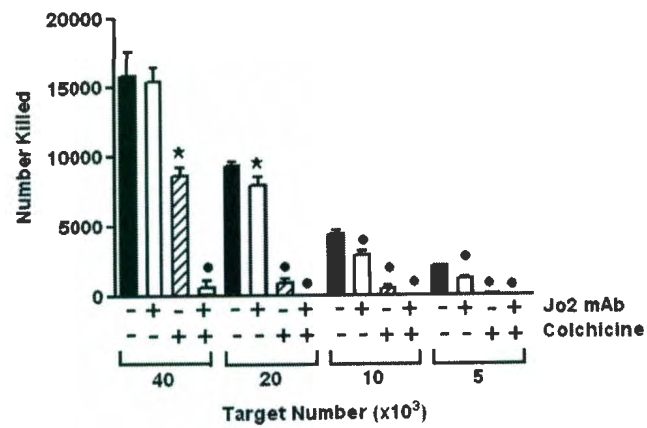
Results were analyzed by one way ANOVA or unpaired Student's-*t* test with Welch's correction using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA).

3.3 RESULTS

3.3.1 Granule exocytosis contributes to hepatocyte killing of CD95-positive cells

We have previously documented that normal hepatocytes transcribe CD95L and can induce death of CD95-bearing cells (Guy et al., 2006). However, pretreatment of CD95L-sensitive P815 target cells with Jo2 mAb, which blocks CD95 (Guy et al., 2006; Hodgson et al., 1999), significantly inhibited but did not completely abrogate the killing (Fig. 3.1A). This suggested that hepatocytes could be equipped with an

Fig. 3.1. Dependence of hepatocyte killing of CD95-bearing target cells on CD95L and microtubule polymerization. WCM-260 hepatocytes and primary human hepatocytes were used as effectors against CD95-positive P815 cell targets in the JAM DNA fragmentation assay. P815 targets were pre-treated (+) or not (-) with the antagonistic anti-CD95 Jo2 mAb before incubation with hepatocytes in either the presence (+) or absence (-) of colchicine (A and B). Data bars represent the mean \pm SEM of three separate experiments each with 4-6 experimental wells per condition tested. Inhibition of cytotoxicity was significant for data bars marked with * at $P < 0.05$ and with • at $P < 0.0001$.

A**WCM-260 : P815****B****Human Hepatocytes : P815**

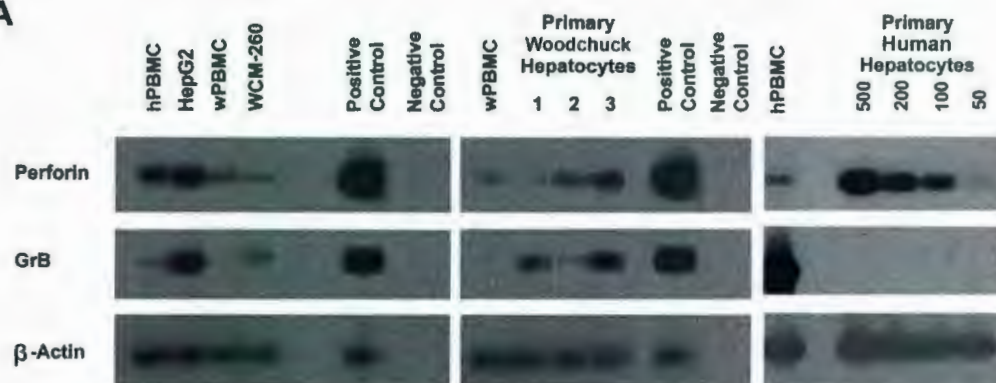
alternate, CD95L-independent cytotoxic mechanism. WCM-260 hepatocyte cytotoxicity against P815 targets was examined in the presence of both anti-CD95 Jo2 mAb and colchicine, an inhibitor of microtubule polymerization. Killing of P815 was significantly reduced ($P < 0.05$) but not abolished by Jo2 mAb (Fig. 3.1A) (Guy et al., 2006). Treatment with both colchicine and Jo2 mAb or colchicine alone eliminated P815 lysis (Fig. 3.1A), suggesting that hepatocytes concomitantly utilize CD95L and granule exocytosis. Primary human hepatocytes used as effectors in similar experiments gave comparable results (Fig. 3.1B). A contribution of alternative cytotoxic mechanisms dependent upon expression of TNF α or TNF-related apoptosis inducing ligand (TRAIL) was excluded since WCM-260 hepatocytes do not transcribe TNF α (data not shown) and P815 cells are resistant to TRAIL-mediated lysis, as reported (Kayagaki et al., 1999; Takeda et al., 2001).

3.3.2 Hepatocytes constitutively transcribe perforin and granzyme B

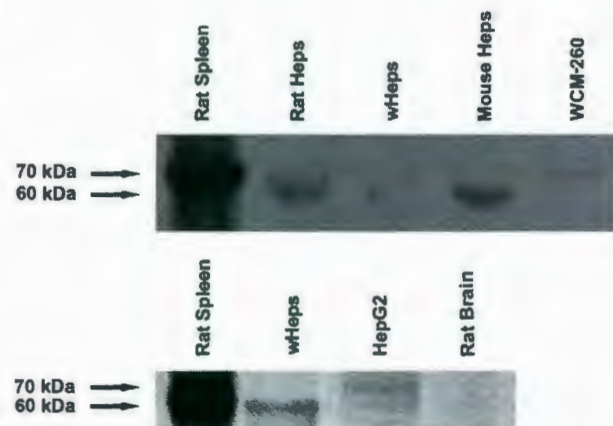
Granule exocytosis-mediated apoptosis is reliant upon expression of perforin and various serine proteases, including granzyme B. As shown in Figure 3.2A, perforin and granzyme B mRNAs were detectable in human HepG2 cells and in woodchuck WCM-260 hepatocytes. Furthermore, perforin mRNA was also consistently detectable in pure primary woodchuck and human hepatocytes (Fig. 3.2A). Woodchuck primary and cultured hepatocytes and human HepG2 cells also were granzyme B mRNA positive (Fig. 3.2A), which was seemingly lacking in human primary hepatocytes (Fig. 3.2A). Absence of granzyme B expression does not, however, preclude perforin direct cytotoxic action, as perforin-dependent yet granzyme

Fig. 3.2. Identification of perforin and granzyme B in hepatocytes. (A) Expression of perforin and granzyme B (GrB) mRNA in human HepG2 cells, woodchuck WCM-260 hepatocytes and in primary hepatocytes isolated from three healthy woodchucks (1-3) detected by RT-PCR using 500 ng total RNA per reaction. Perforin mRNA in normal human hepatocytes was amplified using 500 to 50 ng total RNA per reaction. PBMC from a healthy human (hPBMC) and a healthy woodchuck (wPBMC) and plasmid DNAs served as positive controls. Water instead of cDNA was used as a negative control. β -actin served as a housekeeping gene transcription control. (B) Perforin detection by Western blotting in primary rat, woodchuck (wHeps) and mouse hepatocytes, and in cultured WCM-260 and HepG2 cells. Total protein derived from rat spleen and brain served as positive and negative controls, respectively. The positions of 70-kDa and 60-kDa isoforms of perforin are indicated by arrows. (C) Perforin protein was detected by flow cytometric analysis of WCM-260, HepG2 and primary mouse hepatocytes using perforin-specific antibodies (shaded region) or control rabbit IgG (outlined region). Mouse CTLL-2 cells served as a positive control.

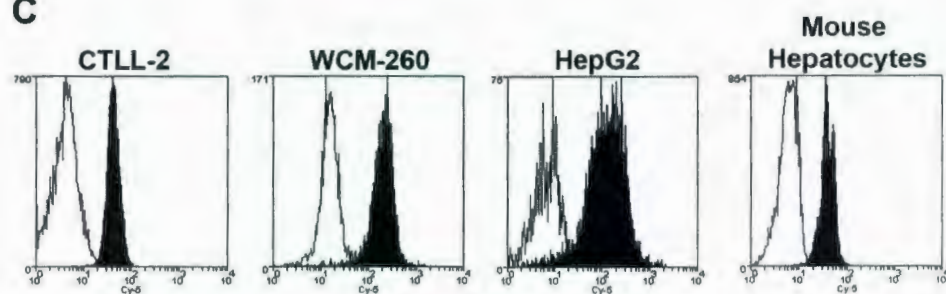
A



B



C



B-independent target cell killing has been reported (Shresta et al., 1997). A possibility that preparations of primary hepatocytes may contain traces of lymphoid cells was excluded based on the absence of CD3 and NKp46 mRNA which are specifically expressed in T, NK and/or NK T cells (data not shown).

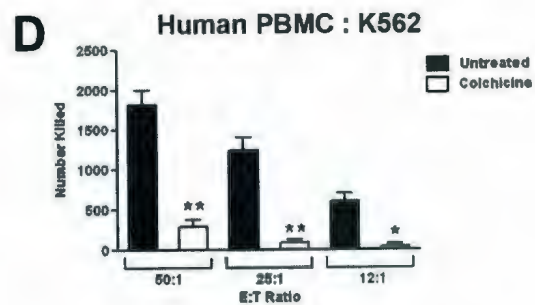
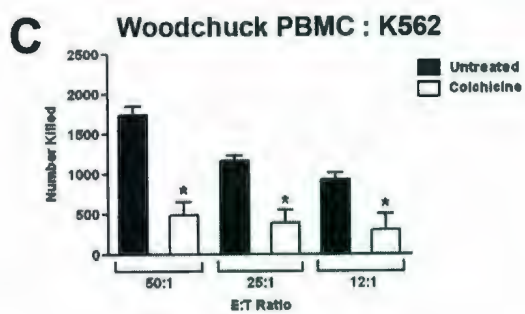
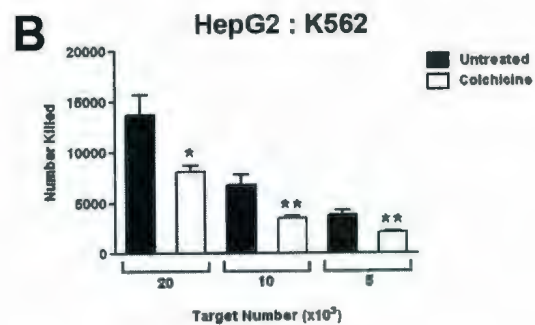
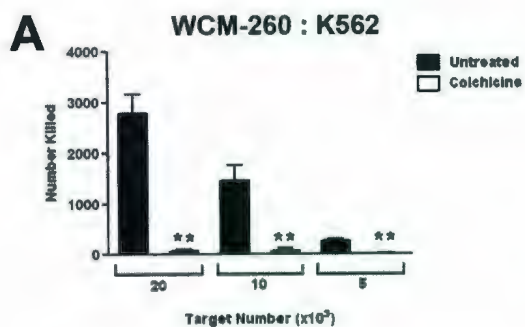
3.3.3 Hepatocytes are endowed in perforin protein

Perforin is initially synthesized as a 65-kd protein which is sequentially glycosylated yielding an intermediate 70- kd protein, followed by proteolytic cleavage resulting in a 60-kd isoform (Uellner et al., 1997). When lysates of cultured hepatocyte lines and primary hepatocytes were analyzed by Western blotting (Fig. 3.2B), the results showed that woodchuck WCM-260 and human HepG2 cells preferentially displayed the 70-kDa perforin isoform, while freshly isolated woodchuck, mouse and rat hepatocytes displayed the 60-kDa isoform (Fig. 3.2B). These results were validated using two different preparations of anti-perforin antibodies (data not shown). Furthermore, the existence of perforin was confirmed by flow cytometry analysis (Fig. 3.2C). A distinct shift in fluorescence intensity after staining with perforin-specific antibodies clearly demonstrated perforin protein presence in both cultured and primary hepatocytes.

3.3.4 Hepatocytes kill CD95-deficient cells

WCM-260 hepatocytes and human HepG2 cells were examined as effectors in JAM DNA fragmentation assays against CD95-deficient K562 cells. Both woodchuck WCM-260 (Fig. 3.3A) and human HepG2 (Fig. 3.3B) eliminated CD95-deficient K562

Fig. 3.3. Hepatocyte killing of CD95-deficient K562 target cells. The cytotoxicity of WCM-260 hepatocytes (A) or HepG2 liver cells (B) against CD95-negative, perforin-sensitive K562 targets was measured by the JAM assay using 6×10^4 effectors and indicated numbers of target cells per assay in the presence of colchicine. PBMC obtained from three healthy woodchucks (C) or three healthy human donors (D) were examined in parallel against 5×10^3 K562 targets at the indicated E:T ratios. Data represent mean values \pm SEM obtained from 3 experiments with 4-6 experimental wells per condition. Data bars marked with ** are significant at $P < 0.01$ and with * at $P < 0.05$ when compared with untreated controls.



cells which was significantly ($P<0.05$ or $P<0.001$) inhibited by colchicine, confirming that target cell death was dependent on microtubule polymerization. Comparable results were obtained when woodchuck PBMC (Fig. 3.3C) or human PBMC (Fig. 3.3D) were used as effectors in control experiments.

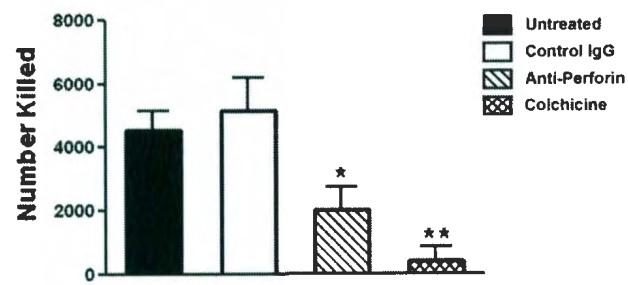
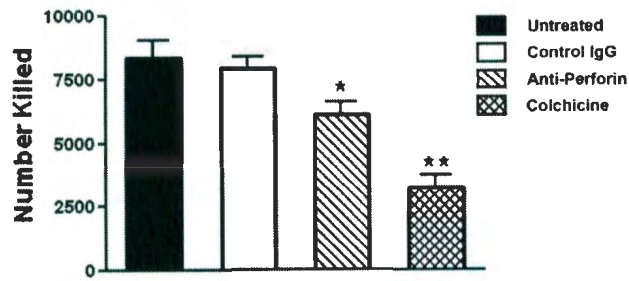
3.3.5 Hepatocyte cytotoxicity is inhibited by anti-perforin antibody

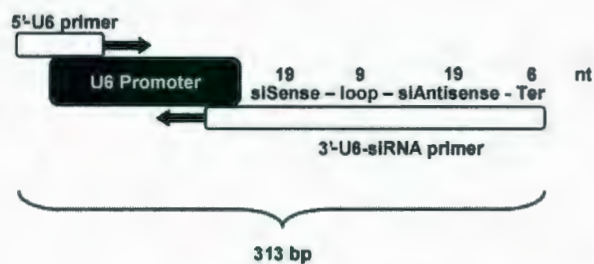
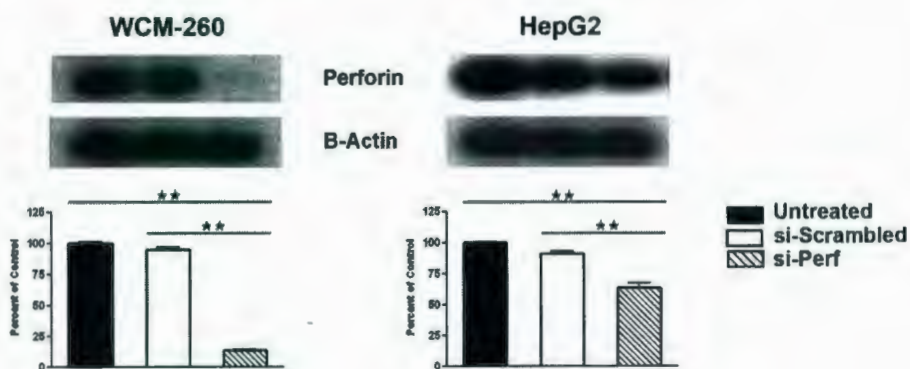
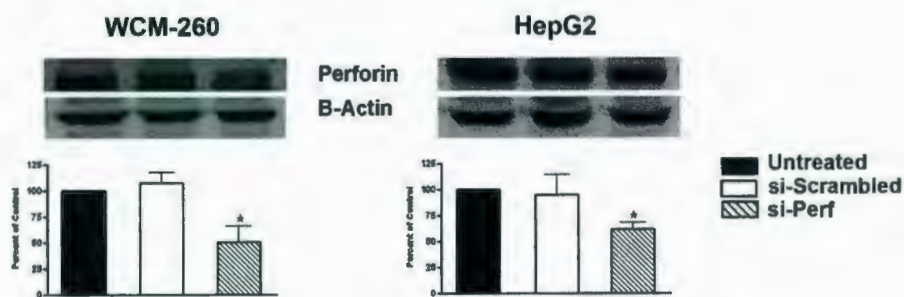
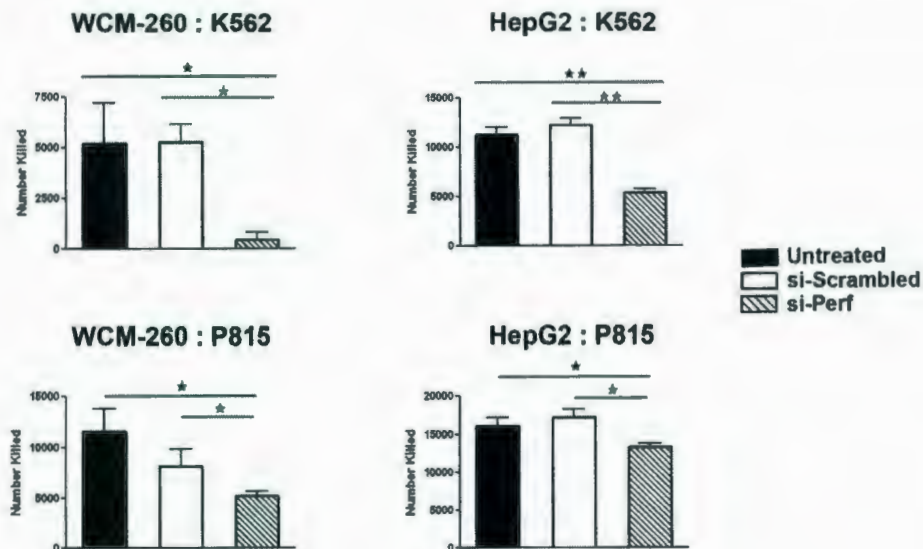
Hepatocyte cytotoxicity was measured in the presence of neutralizing anti-perforin antibodies. Killing of K562 by WCM-260 and HepG2 was significantly reduced in the presence of the antibodies in comparison with either untreated cells or those incubated with a control IgG fraction. Specifically, WCM-260-mediated killing was diminished by 55-60% (Fig. 3.4A), while that induced by HepG2 cells by 24-29% (Fig. 3.4B). Killing of K562 by both WCM-260 and HepG2 was disrupted by colchicine to a significantly greater degree than by anti-perforin antibodies, with approximate reductions of 90% and 60%, respectively (Figs 3.4A and 3.4B).

3.3.6 Perforin-specific shRNA inhibits hepatocyte cytotoxicity

Perforin-specific RNA interference (RNAi) can reduce the cytotoxicity of human NK cells (Ma et al., 2004). Since the sequence of the human perforin gene targeted by RNAi (Ma et al., 2004) is conserved between human, mouse and woodchuck, we employed a PCR-based approach for the generation of this perforin-specific shRNA (see Fig. 3.5A). Transfection with si-Perf effectively reduced the levels of perforin mRNA (Fig. 3.5B) and perforin protein (Fig. 3.5C) in both WCM-260 hepatocytes and HepG2 cells. While the reductions in perforin mRNA and protein expressions were not

Fig. 3.4. Inhibition of hepatocyte killing of CD95-deficient, perforin-sensitive K562 cells by anti-perforin antibodies. WCM-260 hepatocyte (A) or HepG2 liver cell (B) killing was measured by the JAM assay using 6×10^4 effectors and 4×10^4 K562 targets per assay, performed in the presence of neutralizing anti-perforin antibodies, control rabbit IgG or colchicine. Data bars represent the mean \pm SEM of three separate experiments each with 4-6 experimental wells per condition. Inhibition of cell killing is significant for the data bars marked with ** at $P < 0.001$ and with * at $P < 0.05$ relative to untreated or control IgG-treated cells.

A**WCM-260 : K562****B****HepG2 : K562**

A**B****C****D**

as profound in HepG2 cells as in WCM-260 hepatocytes, this was not unexpected since HepG2 cells are known to be difficult to transfect with either plasmid DNA or oligonucleotides. A reduction in perforin mRNA level was also observed in Jurkat cells transfected with si-Perf (data not shown). JAM assays revealed that killing of K562 by WCM-260 or HepG2 was significantly ($P < 0.001$) impaired after their transfection with the si-Perf sequence (Fig. 3.5D). Significant ($P < 0.01$) reductions in target cell killing were also seen when si-Perf-transfected WCM-260 or HepG2 cells were used as effectors against P815 targets (Fig. 3.5D).

3.3.7 Primary hepatocytes kill CD95-deficient cells

To ascertain whether hepatocytes may utilize the perforin pathway *in vivo*, we examined whether primary hepatocytes freshly isolated from mice, healthy woodchucks or a human are competent to kill K562 targets. Figure 3.6A documents that killing of K562 cells by primary mouse hepatocytes was significantly ($P = 0.0003$) reduced in the presence of anti-perforin antibodies but not control IgG. Inclusion of colchicine also meaningfully ($P < 0.0001$) reduced target cell lysis. Transfection of primary mouse hepatocytes with si-Perf, but not with a scrambled shRNA sequence, led to a significant ($P = 0.04$) decrease in perforin protein expression, as evidenced by Western blot analysis (Fig. 3.6B). In addition, JAM cytotoxicity assays revealed that transfection of hepatocytes with si-Perf, but not with a si-scrambled sequence, significantly ($P = 0.0014$) reduced killing of K562 targets by hepatocytes. Figure 3.7 documents further that fresh, primary woodchuck or human hepatocytes not subjected to culture were also effective mediators of CD95-independent cell death. Inclusion of

Fig. 3.6. Killing of CD95-deficient target cells by primary mouse hepatocytes. The cytotoxic potential of hepatocytes freshly isolated from three outbred mice was examined by the JAM DNA fragmentation assay using 6×10^4 hepatocytes and 4×10^4 K562 targets. (A) Hepatocytes and target cells were incubated in the presence or absence of control rabbit IgG, anti-perforin antibodies or colchicine. Reductions in target cell lysis in comparison with untreated or control IgG-treated groups where ** is significant at $P < 0.005$. Decreased perforin protein (B) or K562 target cell killing (C) following transfection of mouse hepatocytes with si-Perf or si-Scrambled. In (B), a representative Western blot demonstrating decreased perforin protein and a graphic presentation of the means \pm SEM of the densitometric values of the signals obtained from three separate experiments after normalization to β -actin protein showed as a percentage of the means given by untreated hepatocytes. In (C), killing of 4×10^4 K562 cells by 6×10^4 untreated or transfected hepatocytes determined by the JAM DNA fragmentation assay. Bars represent mean values \pm SEM obtained from three experiments with 4-12 experimental wells per condition. Differences between experimental conditions were significant at $P < 0.01$ when * or $P < 0.005$ when **.

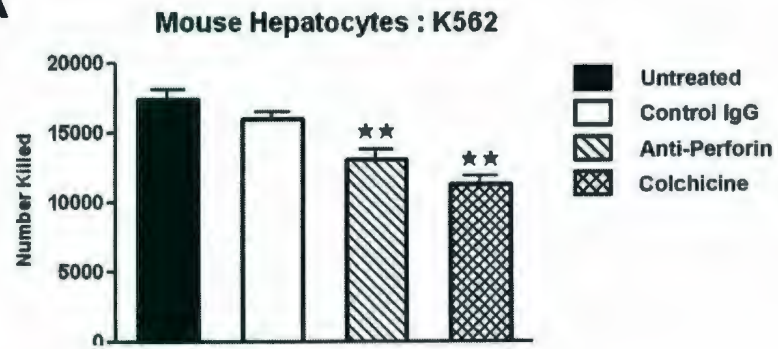
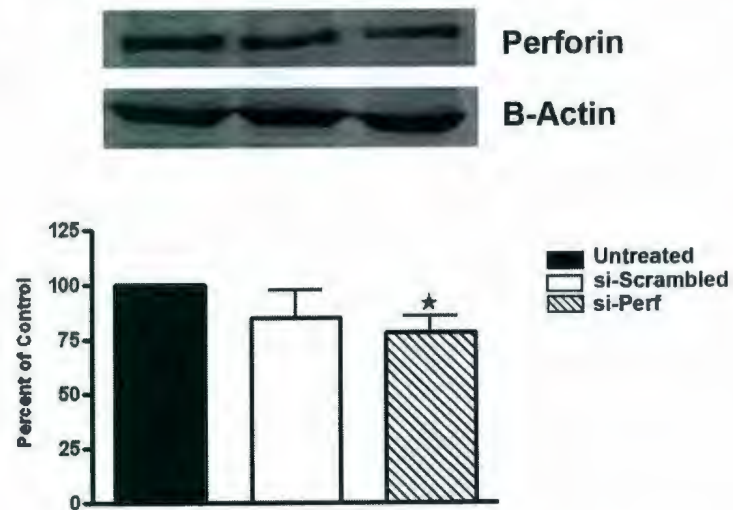
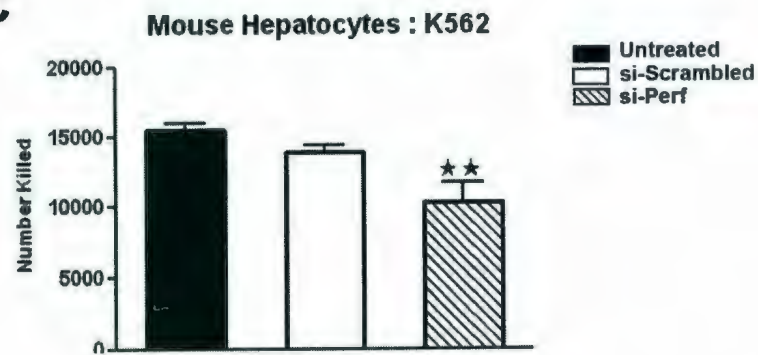
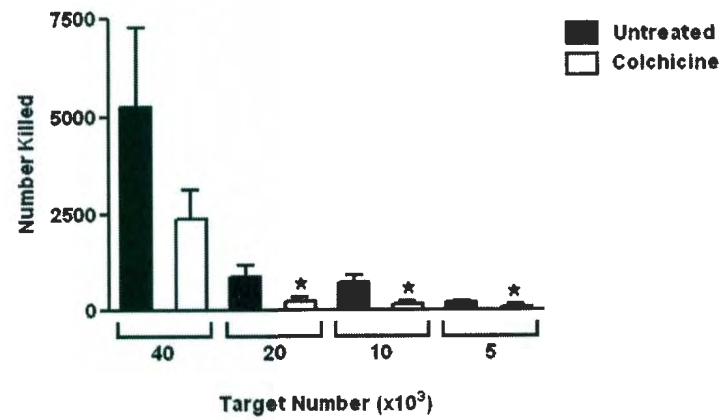
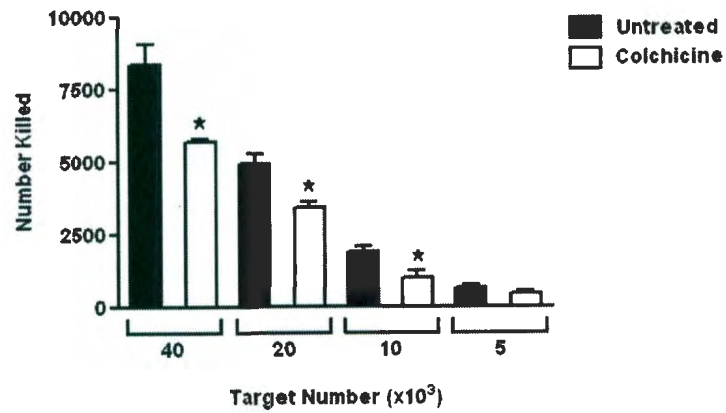
A**B****C**

Fig. 3.7. Killing of CD95-deficient cells by primary woodchuck and human hepatocytes. The cytotoxic capacity of hepatocytes freshly isolated from 3 healthy woodchucks (A) or a healthy portion of a human liver (B) was examined by the JAM DNA fragmentation assay using K562 cells as targets. Colchicine significantly ($P<0.05$) inhibited target cell apoptosis as compared to untreated controls when indicated by *. Data represent mean values \pm SEM with 4-6 experimental wells per condition.

A**Woodchuck Hepatocytes : K562****B****Human Hepatocytes : K562**

colchicine again significantly ($P<0.005$ or $P<0.05$) decreased elimination of K562 targets, confirming a requirement for microtubule polymerization in killing of CD95-negative cells.

3.3.8 Hepatocyte killing of CD95-deficient cells is not modulated by IFN γ

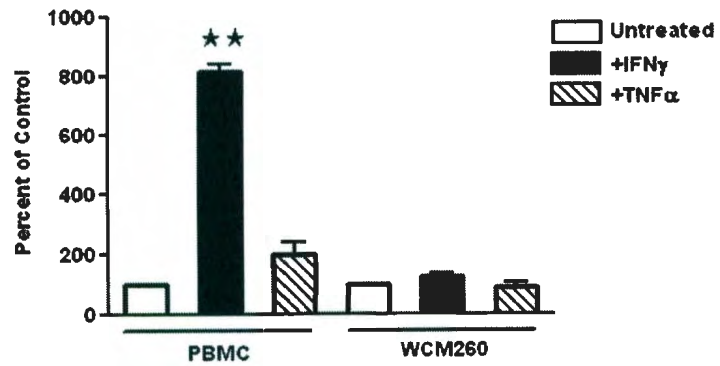
Since IFN γ and TNF α augment hepatocyte CD95L-dependent cytotoxicity (Guy et al., 2006), we investigated if these cytokines also enhance perforin-mediated cell killing. WCM-260 hepatocytes were exposed to either IFN γ or TNF α under conditions determined previously (Guy et al., 2006). It was found that perforin mRNA was significantly ($P<0.001$) upregulated in PBMC in response to treatment with IFN γ , but not with TNF α (Fig. 3.8A). In contrast, the level of perforin mRNA in WCM-260 hepatocytes was not influenced by either IFN γ or TNF α (Fig. 3.8A). Concomitantly, while IFN γ -stimulated PBMC were significantly ($P<0.01$) more cytotoxic than untreated PBMC (Fig. 3.8B), the cytotoxic activity of WCM-260 remained unchanged (Fig. 3.8C). The data suggest that perforin expression could be modulated by IFN γ in a cell type-specific manner.

3.4 DISCUSSION

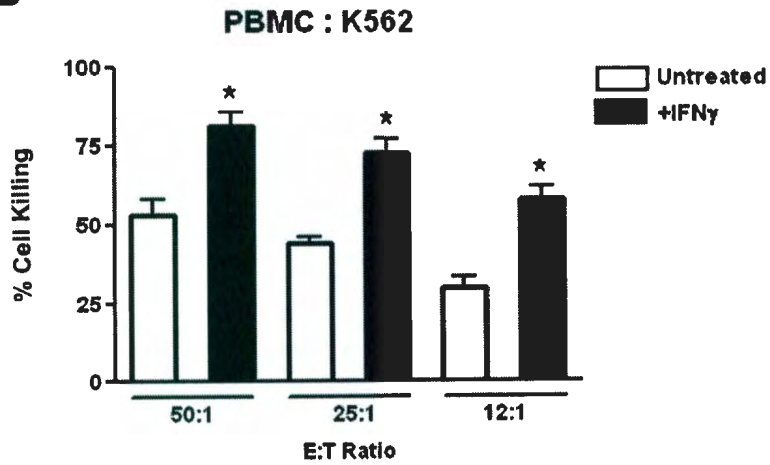
It has become apparent that the liver can *de facto* be considered as an immunocompetent organ and may act as a final repository for activated CD8+ T cells (Crispe et al., 2000). While the exact mechanisms of intrahepatic retention and induction of T cell apoptosis are not defined, hepatocytes can act as cytotoxic effectors and eliminate cell targets via the CD95L-CD95 pathway

Fig. 3.8. IFN γ differentially modulates perforin gene expression in hepatocytes and PBMC. (A) Perforin mRNA expression in normal woodchuck PBMC and WCM-260 hepatocytes following stimulation with 150 U/ml wrIFN γ or 35 U/ml wrTNF α as determined by real time RT-PCR. Data represent the mean \pm SEM of the values obtained from three experiments after normalization to β -actin, and are displayed as a percentage of the means given by untreated PBMC or WCM-260 where ** is significant at $P < 0.001$. The cytolytic activity of IFN- γ -stimulated PBMC (B) or WCM-260 hepatocytes (C) was evaluated against K562 target cells in JAM assay using untreated effectors as controls. Results are shown as mean values \pm SEM from 4-6 experimental wells per each E:T ratio. Data bars marked with * are significant at $P < 0.01$.

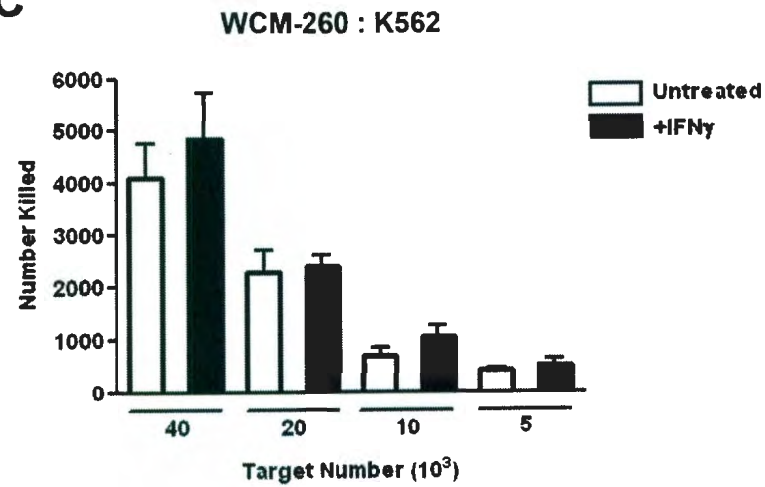
A



B



C



(Guy et al., 2006; Iken et al., 2006). Therefore, hepatocytes may contribute to removal of activated lymphocytes or other cells either passing through or residing in the liver. It is well understood that CTL and other activated lymphoid cells, such as NK T cells, can eliminate targeted cells through CD95L-CD95 interactions or as a result of polarized release of cytolytic granules containing perforin and serine proteases (Barry and Bleackley, 2002). However, the usage of a cytolytic granule exocytosis pathway by non-lymphoid cells has not been previously reported.

Our initial experiments from this study, in which CD95-bearing P815 cells served as targets, clearly demonstrated that killing by hepatocytes was dependent upon both CD95L-CD95 interactions and microtubule polymerization. This was consistent with earlier studies utilizing lymphoid effectors against P815 targets (Hodgson et al., 1999; Spaner et al., 1998). The finding that colchicine alone, in the absence of CD95-blocking Jo2 antibodies, near completely inhibited P815 cell killing suggests that hepatocytes may contain CD95L within cytoplasmic granules, as has been reported for lymphoid effector cells (Kojima et al., 2002). Previous reports implied that hepatocytes do not express perforin or granzyme B, as determined by immunohistochemical or *in situ* hybridization methods (Ibuki et al., 2002; Tordjmann et al., 1998). However, both cultured hepatocytes and, more importantly, primary hepatocytes derived from rodents and a human express perforin and, in the case of rodent hepatocytes and human HepG2 cells, granzyme B mRNAs when analyzed by RT-PCR, as our current study showed. Furthermore, immunoblot and flow cytometry analyses of the perforin mRNA-positive cells confirmed that perforin protein is normally displayed by hepatocytes. Interestingly, hepatocyte cell lines showed a

perforin isoform corresponding to a 70-kDa intermediate form, whereas a mature 60-kDa isoform was detected in primary hepatocytes and splenic tissue. Although the significance of this discrepancy is unclear, both cultured and primary hepatocytes efficiently killed CD95-deficient K562 target cells.

Killing of CD95-negative cells by hepatocytes was significantly reduced following inhibition of microtubule polymerization. These results provide the first experimental evidence that hepatocytes can be cytotoxic in a manner independent of CD95-activation. Additional experiments with neutralizing anti-perforin antibodies confirmed that killing of CD95⁺ targets was perforin-dependent. However, anti-perforin antibodies were less inhibitory than colchicine. This finding is not unexpected since colchicine is highly efficient in preventing granule exocytosis and release of perforin, while antibodies act following degranulation and this process is certainly less effective in neutralizing perforin and may require a much higher concentration of anti-perforin antibodies than that used.

Our results have also firmly demonstrated that perforin-specific shRNA, targeting a sequence previously shown to abrogate NK cell cytotoxicity (Ma et al., 2004), impaired the ability of both cultured and freshly isolated primary hepatocytes to kill cells. Silencing of perforin gene expression in WCM-260 and HepG2 cells also significantly reduced killing of CD95-positive targets, which is consistent with our earlier findings (see Figs 3.1 and 3.3A) indicating that the apoptosis of P815 was at least in part, perforin-dependent.

The levels of perforin mRNA expression and CD95-independent cell killing were not influenced by exposure of hepatocytes to IFN γ or TNF α . This is in contrast

with the augmented transcription of perforin and the increased apoptosis of target cells after exposure of lymphoid cells to IFN γ . This discrepancy requires further investigation, however, differential regulation of perforin expression has also been postulated for NK cells and T lymphocytes (Lacorazza et al., 2002). Opposing effects of IFN γ on the activity of the cytotoxic pathways utilized by two cell types closely interacting with each other under physiological or pathological conditions suggest an important role for this cytokine in altering hepatocyte cytotoxic potential and in modulating hepatocyte interactions with lymphoid and other cells.

Autocidal death of CD8 $^{+}$ T cells requires perforin (Spaner et al., 1998), while perforin-deficient mice infected with lymphocytic choriomeningitis virus (LCMV) show accumulation of virus-specific CD8 $^{+}$ T cells in lymphoid organs and the liver (Matloubian et al., 1999), implicating the importance of perforin in the contraction of CTL. Constitutive expression of perforin may suggest that hepatocytes can contribute to removal of activated CD8 $^{+}$ T cells. The contact between hepatocytes and cells predestined for elimination could be facilitated by interaction between terminally desialiated proteins exposed on target cells and hepatocyte ASGPR. Neuraminidase treatment of lymphocytes has been shown to enhance their retention in the liver (Samlowski et al., 1984), while we have observed that treatment of P815 and K562 cell targets with neuraminidase enhances their killing by hepatocytes (unpublished observation).

On the other hand, expression of CD95L may also imply the potential of hepatocytes to eliminate CD4 $^{+}$ T cells (Suzuki and Fink, 2000). In the case of hepatic inflammation, which is normally accompanied by augmented levels of intrahepatic

IFN γ and/or TNF α (Hodgson and Michalak, 2001), hepatocyte CD95L.-dependent killing might be heightened resulting in the preferential elimination of CD4 $^{+}$ T cells. In viral hepatitis, this may constrain a collaborative networking between virus-specific CD4 $^{+}$ and CD8 $^{+}$ T cells, and differentially modulate clearance of virus and the outcome of hepatitis. At this stage, this sequence of events remains hypothetical, but our results provide the framework for further testing of this concept.

Overall, we have shown that hepatocytes, which are a non-lymphoid cell type, are functionally competent to eliminate cells by a perforin-mediated mechanism. Our study strengthens the notion that hepatocytes are active contributors to both physiological and pathological processes occurring in the liver (Guy et al., 2006). The cytotoxic activity of hepatocytes may influence the pathogenicity of liver diseases, including those caused by hepatotropic viruses.

**CHAPTER FOUR: HEPADNAVIRAL INFECTION AUGMENTS
HEPATOCTE CYTOTOXICITY MEDIATED BY BOTH CD95 LIGAND AND
PERFORIN-DEPENDENT PATHWAYS**

4.0 SUMMARY

We recently demonstrated that normal hepatocytes express CD95L, can eliminate contacted cells via the CD95L-CD95 pathway, and that this cytotoxic activity can be augmented by IFN γ . We have also documented that hepatocytes are endowed in perforin and granzyme B, and can extinguish other cells via the granule exocytosis mechanism. The current study aimed to assess whether hepadnavirus infection modifies hepatocyte-mediated cell killing during active infection and after recovery from self-limited acute hepatitis (SLAH) in the woodchuck model of hepatitis B. We discovered that hepatocytes isolated during both chronic viral hepatitis and following SLAH display significantly greater cytotoxicity towards cell targets than those from healthy animals, and that both hepatocyte CD95L- and perforin-dependent cell killing are augmented. In supplementary experiments, woodchuck hepatocytes transfected with WHV X gene, but not those transfected with complete WHV DNA or virus envelope or core gene, expressed substantially higher levels of CD95L and perforin mRNAs, and killed cell targets more efficiently. Also, exposure of hepatocytes to IFN γ , except those transfected with WHV X gene, profoundly enhanced their cytotoxicity. In conclusion, hepatocyte CD95L and perforin-mediated cytotoxicity is significantly augmented during natural hepadnaviral infection and after resolution of hepatitis. This could be a consequence of increased intrahepatic production of IFN γ .

due to virus-induced liver inflammation, although a role for defective virus replication associated with over-expression of virus X protein can not be entirely excluded.

4.1 INTRODUCTION

The liver performs multiple immune-like functions, including the removal of aberrant cells, elimination of pathogens, and induction of peripheral immunotolerance (Racanelli and Rehermann, 2006). This organ also appears to be the primary site of elimination of T cells, particularly activated CTL (Crispe et al., 2000). Hence, the liver is not only a component of the innate immune defense but it may modulate effectiveness of T lymphocyte responses, of which those directed against antigens critical for the development of hepatic injury might be of a particular importance. Although the data accumulated imply that the above processes could be to a large degree mediated by LSEC (Diehl et al., 2007) and intrahepatic immune cells, especially macrophages (Mehal et al., 2001), a contribution of hepatocytes also is highly probable due to the structure of hepatic vasculature (Warren et al., 2006) and to the recently discovered hepatocyte cytotoxic effector activity (Guy et al., 2006). In this regard, it was found that normal primary hepatocytes as well as hepatocyte lines constitutively express CD95L and they can eliminate other cells via the CD95L-CD95 mechanism. As well, hepatocyte CD95L expression and CD95-dependent cell killing can be significantly augmented *in vitro* following exposure to IFN γ and, under certain circumstances, TNF α (Guy et al., 2006). Furthermore, it was recently found that hepatocytes also express perforin and can utilize an exocytic pathway to kill both CD95-deficient and CD95-bearing cells (Guy et al., 2008, in press, see Chapter 3).

Interestingly, in contrast to lymphocytes, hepatocyte perforin-dependent killing is not influenced by IFN γ or TNF α . Taken together, hepatocytes, similarly as lymphocytes, are endowed in two distinct cytotoxic effector mechanisms. However, in contrast to lymphocytes, hepatocyte cytotoxicity mediated by these pathways could be differentially modulated by the local cytokine milieu. The *in vivo* relevance of these findings may pertain not only to fratricidal cell death during the development and progression of liver disease, but implicate further an active cytotoxic role for hepatocytes as they may interact with lymphocytes and other cells either trafficking through or infiltrating the diseased liver. Furthermore, the notion that the liver eliminates activated lymphocytes and maintains a uniquely tolerogenic environment might in part be related to the cytopathic capability of hepatocytes.

Antiviral specific T cell responses, especially that mediated by CTL, are thought to be of primary importance in the control of HBV infection (reviewed in (Guidotti and Chisari, 2000)). The same cytotoxic activities, as well as pro-inflammatory cytokines released are main contributors to liver damage manifesting as hepatitis. The activated CTLs may also play a role in non-cytopathic clearance of hepadnavirus through production of IFN γ and TNF α and by a feedback stimulation of other immune cells, such as NK, NK T cells and macrophages. It has been postulated that while CTL-mediated, perforin- and CD95L-dependent hepatocyte injury is predominantly responsible for induction of hepatitis, downregulation of virus replication in hepatocytes is chiefly a consequence of intrahepatically produced IFN γ and TNF α (Guidotti et al., 1996; Kakimi et al., 2000). Moreover, while AH and recovery from acute infection are associated with vigorous, polyclonal and

multispecific CTL and T helper type 1 responses to HBV antigens, CH is accompanied by a low number of circulating and intrahepatic virus-specific CD8⁺ and CD4⁺ T cells. The reason behind this remains unknown, but an active contribution of hepatocytes has to be considered in the context of their cytotoxic potency recently identified.

IFN γ and TNF α have been proven to be indispensable for recovery from hepadnaviral infection, both in experimental HBV infection in chimpanzees and in woodchucks infected with WHV (Hodgson and Michalak, 2001). Upregulated hepatic expression of these cytokines was also found, although to a lesser extent than in CH, to continue for years after resolution of SLAH in the context of persistent low level replication of WHV and intermittent minimal to moderate inflammatory changes in hepatic tissue of the woodchucks recovered (Hodgson and Michalak, 2001). It was postulated that antiviral cytokines, in particular IFN γ , may play a central role not only in recovery from AH but also in the long-term control of occult hepadnavirus infection in the liver (Hodgson and Michalak, 2001). Since IFN γ , and under some conditions TNF α , efficiently upregulates hepatocyte CD95L-dependent cytotoxicity, this activity may contribute to a dynamic equilibrium in which the persistently multiplying virus is kept under confinement through immune pressure exerted, among others, by virus-specific CTL, while the cytokine-triggered hepatocytes may defend themselves by eliminating these highly cytopathic cells which are brought in contact with their surface.

To recognize the cytotoxic capabilities of hepatocytes in the course of hepadnaviral infection and to learn whether the possibility outlined above may exist, we investigated primary hepatocytes freshly isolated from woodchucks with CH or

experimentally induced SLAH. We have found that the potential of hepatocytes to kill other cells is consistently augmented both in CH and long after recovery from SLAH. The increased killing was unlikely a direct consequence of viral replication in hepatocytes, as *in vitro* experiments with recombinant WHV in cultured hepatocytes suggested, but rather a result of enhanced hepatic IFN γ production due to active or residual inflammation.

4.2 MATERIALS AND METHODS

4.2.1 Cell lines

CD95-bearing murine mastocytoma P815 (ATCC TIB-64; American Type Culture Collection, Rockville, MD) and lymphoma LS102.9 cells (ATCC HB-97), and CD95-deficient human chronic myelogenous leukemic K562 cells (ATCC CCL-243) were cultured as described (Guy et al., 2006; Hodgson et al., 1999). Woodchuck WCM-260 hepatocyte cell line was established from the liver of a healthy woodchuck and the cells were phenotypically characterized and maintained as reported (Churchill and Michalak, 2004; Diao et al., 1998). The parental WCM-260 was utilized to derive stably transfected cell lines expressing a complete WHV genome or WHV individual genes (Wang and Michalak, 2006).

4.2.2 WHV infection

Eighteen adult woodchucks were investigated. Five were healthy WHV-naïve animals which served as controls. Seven of the remaining 13 (Table 1) were infected with 1.1×10^{10} DNase-protected virus genome equivalents (vge) of WHV/tm2 inoculum (GenBank accession number AY628095) (Coffin et al., 2004), as reported (Michalak et

al., 1999). The status of WHV infection was monitored by testing serial serum samples for WHsAg, anti-WHs and anti-WHc with assays reported before (Coffin et al., 2004; Michalak et al., 1999). SLAH was diagnosed in all 7 animals. Six other woodchucks had serum WHsAg-positive CH acquired in the wild prior to arrival to our colony (Table 1). Liver histology was examined in serial biopsies and at the time of hepatocyte isolation, following previously established criteria (Hodgson and Michalak, 2001). The examination at the time of autopsy confirmed diagnosis of either CH or resolved AH with minimal to moderate inflammatory changes, as reported (Coffin et al., 2004; Hodgson and Michalak, 2001; Michalak et al., 1999) (Table 1).

4.2.3 Primary hepatocytes

Hepatocytes were immediately isolated by two-step collagenase microperfusion of liver tissue fragments obtained from each animal by procedures previously reported (Churchill and Michalak, 2004). Hepatocytes were at least 98% pure by phase-contrast microscopy and viability was consistently determined to be greater than 80% as determined by trypan blue exclusion. Potential contaminations with T lymphocytes, NK cells and NK T cells were excluded by analysis of total RNA extracted from hepatocyte preparations for expression of woodchuck CD3 and NKp46 using RT-PCR as reported (Guy et al., 2006; Hodgson and Michalak, 2001). Animal experimental protocols were approved by the Institutional Presidents' Committee on Animal Bioethics and Care.

Table 4.1. Serological and histological characteristics of WHV infection in animals with self-limited acute hepatitis or chronic hepatitis investigated in this study

Status of WHV Infection and Animal No./Sex	Observation period (mo)*	Serum WHsAg positivity	Duration of WHsAg (mo)	Anti-WHe positivity	Duration of anti-WHe (mo)	Histological degree of hepatitis
Resolved hepatitis						
1/F	5.5	+	1	+	4.5	0
2/F	8	+	1	+	7	0.5
3/F	6	+	2.75	+	4.5	0.5
4/F	21	+	0.75	+	19.5	0
5/F	8.25	+	2	+	6	0.5
6/F	31	+	2.5	+	29.5	0.5
7/M	10.25	+	1	+	5.5	1
Chronic hepatitis						
8/F	33.75	+	33.75	n.t.	n.t.	2
9/F	6.5	+	6.5	n.t.	n.t.	2
10/M	12.5	+	12.5	n.t.	n.t.	1
11/M	7	+	7	n.t.	n.t.	1.5
12/M	25.25	+	25.25	n.t.	n.t.	2
13/M	33	+	33	n.t.	n.t.	1.5

mo, months; F, female; M, male; n.t., not tested.

* from the time of inoculation with WHV for animals with resolved hepatitis or the

time of the arrival to colony for animals with serum WHsAg-positive chronic hepatitis.

4.2.4 Hepatocytes transfection with WHV DNA

Hepatocytes stably expressing either a complete WHV genome, individual WHV genes or empty vector (control) were established using WCM-260 hepatocytes, as recently reported (Wang and Michalak, 2006). Plasmids carrying WHV gene sequences encoding functionally distinct proteins were constructed using the eukaryotic expression vector pcDNA3.1 (Invitrogen, Carlsbad, CA). A plasmid containing approximately 1.1 times the length of the WHV genome (p-WHV) was produced by a two-step PCR amplification of wild-type WHV/tm3 DNA sequence (GenBank accession number AY334075) (Michalak et al., 2004). To examine whether individual WHV proteins could influence the cytotoxic capacity of hepatocytes, WCM-260 cells stably expressing pcDNA3.1 plasmids containing complete WHV envelope large (pre-S1) protein (p-WHs), core protein (p-WHc) or X protein (p-WHx) were generated (Wang and Michalak, 2006). The display of WHV translation products or release of infectious virions by hepatocytes were ascertained as reported (Wang and Michalak, 2006).

4.2.5 Hepatocyte treatment with IFN γ

WCM-260 hepatocytes stably transfected with either complete WHV genome, virus individual genes or with empty vector were exposed to bioactive rwIFN γ generated in the baculovirus expression system as reported (Wang and Michalak, 2005). The cells were treated for 18 h with 150 U/ml of rwIFN γ , while untreated controls were cultured under the same conditions in the absence of the cytokine (Guy et al., 2006).

4.2.6 RNA extraction and reverse transcription

Total RNA was extracted from 2×10^5 or 1×10^6 cultured or primary hepatocytes or from control HepG2 cells, and from 100 mg of woodchuck liver tissue using Trizol reagent (Invitrogen). Potential DNA contamination was removed using RNase-free, DNase digestion kit (Sigma-Aldrich, Oakville, Ontario, Canada). RNA (2 μ g) was reverse transcribed to cDNA in a 20- μ l reaction volume, as described (Guy et al., 2006).

4.2.7 Real-time RT-PCR

Expression of CD95L, CD95, perforin, granzyme B and β -actin in primary and cultured hepatocytes was quantified by real time RT-PCR employing the LightCycler Faststart Master SYBR I kit (Roche Diagnostics, Laval, Quebec, Canada) and the Roche LightCycler (Roche Diagnostics), as recently described (Guy et al., 2006). Woodchuck CD3, IFN γ and TNF α mRNA were quantified in liver tissue using primers and real-time RT-PCR conditions reported elsewhere (Wang et al., 2007). Serial dilutions containing known copy numbers of the respective recombinant gene fragments were included as quantitation standards. The copy numbers in test samples were determined with LightCycler quantification software (Roche Diagnostics) and were normalized against expression of β -actin, as described (Guy et al., 2006).

4.2.8 Preparation of target cells

P815, LS102.9 and K562 cells were subcultured to a density of 5×10^6 cells/ml and labeled with 50 μ Ci of 3 H-thymidine (Perkin Elmer, Wellesley, MA) for 18 h prior

to the JAM cytotoxicity DNA fragmentation assay, as reported (Guy et al., 2006). The cells were washed 3 times in Hanks' balanced salt solution (Invitrogen), re-suspended in RPMI-1640 medium, and immediately used as targets.

4.2.9 JAM Cytotoxicity (DNA fragmentation) assay

Primary fresh woodchuck hepatocytes were aliquoted at 6×10^4 /well in 200- μ l volumes in 96-well flat-bottom cell culture plates, as reported (Guy et al., 2006). Stably transfected WCM-260 hepatocytes were grown to confluence ($\sim 6 \times 10^4$ cells/well) in the same type of plates. ^3H -thymidine-labeled P815, LS102.9 or K562 cells were added at 4×10^4 , 2×10^4 , 1×10^4 , and 5×10^3 into 4-8 experimental wells each, yielding final effector : target (E:T) ratios of 12:1, 6:1, 3:1, and 1.5:1, respectively. Plates were centrifuged for 5 minutes at $45 \times g$ and incubated at 37°C in a humidified 5% CO_2 atmosphere for 18 h. Well contents were harvested onto glass fibre mats (Perkin Elmer) using a 96-well harvester (Tomtec, Hamden, CT). Counts per minute (cpm) were measured using a Top10 beta counter (Becton Dickinson, San Diego, CA) and percent lysis was determined as reported (Guy et al., 2006).

4.2.10 Inhibition of hepatocyte-mediated cytotoxicity

Jo2 mAb (Becton Dickinson), which blocks CD95 on P815 cells without inducing lysis, has been shown to effectively abrogate hepatocyte- and lymphoid cell-mediated killing of P815 target cells (Guy et al., 2006; Hodgson et al., 1999). This mAb was used to confirm specificity of hepatocyte CD95L-mediated killing. Inhibition of microtubule-dependent granule release was facilitated using 1 mM

colchicine (Sigma-Aldrich) to confirm perforin contribution to hepatocyte cell killing, as described (Hodgson et al., 1999).

4.2.11 Statistical analyses

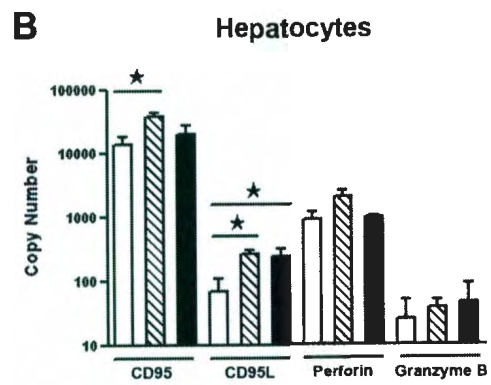
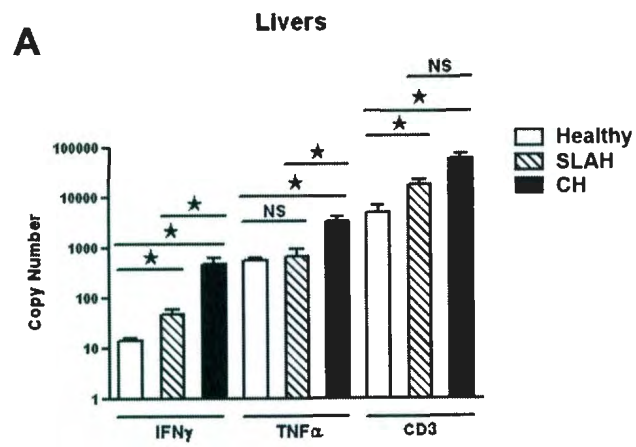
Results were analyzed, where appropriate, by one way ANOVA or unpaired Student's-*t* test with Welch's correction using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA). Differences between experimental conditions were considered to be significant when two-sided *P* values were less than 0.05.

4.3 RESULTS

4.3.1 Hepatocyte cytotoxic phenotype in WHV infection

It has been shown that augmented hepatic IFN γ , TNF α , as well as CD3 expression accompanies not only active liver inflammation, which is a hallmark of CH, but also, although to a much lesser extent, residual inflammation persisting after resolution of AH in woodchucks (Hodgson and Michalak, 2001). However, it remained unknown whether hepadnaviral infection modifies or not, expression of genes affiliated with hepatocyte cytotoxic activity. To address this, transcription of IFN γ , TNF α and CD3 in the livers and the genes encoding effector molecules of the CD95L-CD95 and perforin pathways in hepatocytes were quantified in woodchucks with SLAH or CH and in healthy animals (Fig. 4.1). As expected, CH was accompanied by histologically evident necroinflammation (Table 4.1) and significantly ($P<0.01$) enhanced intrahepatic expression of IFN γ , TNF α and CD3 mRNA, while minimal inflammatory alterations occurring after SLAH (Table 4.1) were associated with

Fig. 4.1. Expression of selected cellular genes known to be associated with progressing liver inflammation and those encoding molecules affiliated with hepatocyte cytotoxic activity. The mRNA levels of the indicated genes were quantified by real-time PCR in the total liver tissue (A) or in essentially pure hepatocytes (B) from woodchucks convalescent from SLAH (n=7) or with progressing CH (n=6) and WHV-naïve, control animals (n=5). Transcription of each gene was analyzed in triplicate in both the livers and hepatocytes preparations obtained from each animal analyzed and was normalized against expression of woodchuck β -actin, as a housekeeping gene. Data are presented as mean copy number \pm SEM. Expression levels significantly different at $P < 0.05$ were marked with *.



significant ($P<0.01$) increases of hepatic IFN γ and CD3, but not TNF α mRNA levels when compared to those in healthy woodchucks (Fig. 4.1A). There also were significant differences ($P<0.05$) in the hepatic expression of IFN γ and TNF α , but not CD3, between animals with SLAH and CH (Fig. 4.1A).

Hepatocytes from animals convalescent from SLAH or with CH transcribed CD95L at significantly ($P<0.01$) greater levels than those from healthy controls (Fig. 4.1B). It is of note that CD95 expression was also augmented ($P<0.01$) in hepatocytes after SLAH. In contrast, perforin and granzyme B mRNA levels were not meaningfully different, although the perforin expression tended to be greater in hepatocytes from animals with SLAH than in those with CH or from healthy woodchucks (Fig. 4.1B). Analysis of the data on the expression of the same genes in hepatocytes purified from 13 additional animals with SLAH did not change the validity of the above observations (data not shown).

4.3.2 Resolution of hepatitis is followed by persistently augmented hepatocyte cytotoxicity

Since hepatocytes obtained after resolution of SLAH transcribed significantly more CD95L and also tended to express more perforin mRNA than those from healthy controls (Fig. 4.1B), it was of interest to assess whether their cell killing capacity was also augmented. For this purpose, hepatocytes were purified from three pairs of woodchucks, wherein each pair consisted of one animal with SLAH and one healthy animal. Liver perfusion and hepatocyte isolations for each pair were performed in parallel and hepatocyte cytotoxicity was measured simultaneously in the same JAM

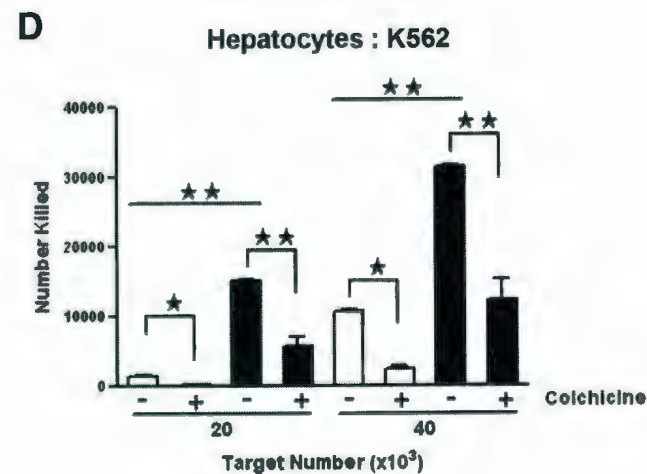
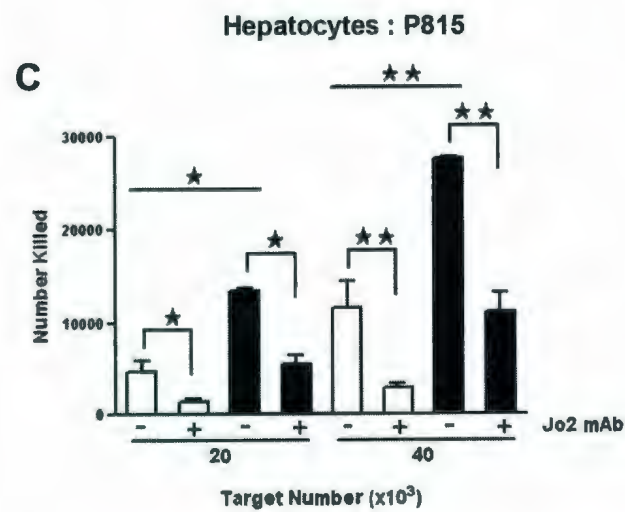
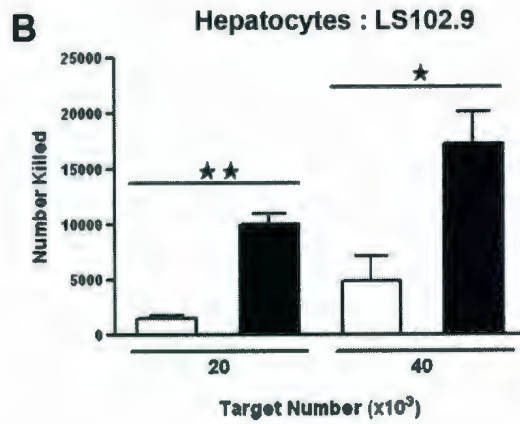
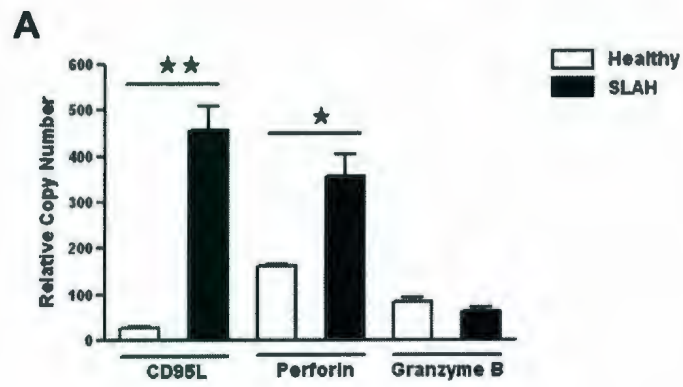
DNA fragmentation assay. The RT-PCR analysis of these three animal pairs showed that hepatocytes from recovered animals exhibited not only significantly greater levels of CD95L mRNA ($P<0.001$) but also perforin mRNA ($P<0.05$), which corresponded to 17.5-fold and 2.2-fold increases, respectively, compared to hepatocytes from healthy controls (Fig. 4.2A). In agreement with the RT-PCR results, the same hepatocytes also were significantly more cytotoxic toward CD95-bearing LS102.9 (Fig. 4.2B) and P815 (Fig. 4.2C) cell targets. The contribution of the CD95L-CD95 pathway was confirmed by pretreatment of P815 cells with an antagonistic CD95 Jo2 mAb (Fig. 4.2C).

Furthermore, the increased perforin expression in hepatocytes from animals convalescent from SLAH (Fig. 4.2A) correlated with augmented killing of CD95-deficient, perforin-sensitive K562 cells (Fig. 4.2D). The involvement of microtubules in this process, characterizing perforin-mediated cell killing, was ensured by significant ($P<0.001$ or $P<0.05$) reductions in K562 cell death in the presence of colchicine, an inhibitor of microtubule polymerization (Fig. 4.2D).

4.3.3 Comparable hepatocyte cytotoxic activity accompanies CH and convalescent period after SLAH

Despite evident hepatic inflammation and significantly greater expression of IFN γ , TNF α and CD3 in the livers of animals with CH than in those with SLAH (Fig. 4.1A), hepatocytes isolated from woodchucks with CH showed the levels of CD95L, perforin or granzyme B mRNA comparable to those in hepatocytes from woodchucks with resolved AH (Fig. 4.1B). To assess the cytotoxic potential of hepatocytes from these two stages of WHV infection, hepatocytes were isolated in pairs from 4

Fig. 4.2. The resolution of acute WHV hepatitis is accompanied by increased hepatocyte cytotoxic activity. Hepatocytes isolated in parallel from three pairs of healthy animals (n=3) and woodchucks who spontaneously resolved SLAH (n= 3) were analyzed for expression of the cytotoxicity-affiliated genes by real-time RT-PCR (A) and for the ability to kill CD95-bearing LS102.9 (B) and P815 (C) cells, and CD95-deficient K562 cell targets (D) in JAM DNA fragmentation assays. Expression of each gene tested (A) was analyzed in triplicate in each hepatocyte preparation and normalized against β -actin. The CD95-specific Jo2 mAb was applied to abrogate CD95L-CD95-mediated killing of P815 cell targets (C), while colchicine was used to disrupt perforin-mediated, microtubule-dependent killing of CD95-deficient K562 cells (D). The results of JAM DNA fragmentation assays are shown as mean values \pm SEM with each experiment performed with 4-8 experimental wells per condition. Differences in the levels of gene expression (A) or in the cytotoxic capacity (B-D) between hepatocytes isolated from animals recovered from SLAH and healthy woodchucks were significant for * at $P<0.05$ and for ** at $P<0.001$. Jo2 mAb or colchicine-dependent inhibition of cell killing was significant in comparison with untreated controls for * at $P<0.05$ and for ** at $P<0.001$.



woodchucks, each pair comprised of one convalescent and one animal with CH. The RT-PCR analysis showed again (see Fig. 4.1B) no differences in the hepatocellular levels of CD95L, perforin or granzyme B gene expressions between SLAH and CH (Fig. 4.3A). Similarly, hepatocytes from both infection groups killed CD95-bearing and CD95-deficient target cells to the comparable degrees (Fig. 4.3B), suggesting that hepatocyte CD95L- and perforin-dependent cytotoxicity were similarly augmented in these two contrastingly different forms of hepadnaviral infection.

4.3.4 Transfection with WHV X gene but not with complete WHV DNA modifies hepatocyte cytotoxic activity

Our previous findings demonstrated that IFN γ and, to a lesser extent, TNF α upregulate hepatocyte expression of CD95L and enhance hepatocyte CD95L-CD95-mediated cell killing (Guy et al., 2006), while having no effect on the transcription of perforin gene and rate of perforin-mediated cytotoxicity (Guy et al., 2008, in press, see Chapter 3). To test whether expression of the complete hepadnavirus genome or genes encoding individual WHV proteins, in the absence of liver inflammation, may influence hepatocyte cytotoxic activity, the cell killing potential of cultured hepatocytes transfected with whole WHV DNA or individual WHV genes was examined. Real-time RT-PCR analysis revealed that cultured woodchuck hepatocytes stably expressing complete WHV DNA or those translating WHV pre-S1 (large) envelope or WHV core protein do not differ in the levels of CD95L or perforin mRNAs when compared with hepatocytes transfected with empty vector (Fig. 4.4A). However, interestingly, transcription of the WHV X gene significantly ($P<0.01$) upregulated both CD95L and

Fig. 4.3. Expression levels of the cytotoxicity-affiliated genes and the cell killing potency displayed by hepatocytes obtained after resolution of acute WHV infection and during active chronic WHV hepatitis. Primary hepatocytes were isolated from animals with CH (n=2) and with past SLAH (n=2). The gene expressions quantified by real-time RT-PCR were normalized against expression of β -actin (A). The cytotoxic capacity of hepatocytes was examined by JAM DNA fragmentation assays using CD95-bearing LS102.9 and P815 cells and CD95-deficient K562 cell targets (B). Results are shown as mean values \pm SEM with each experiment performed with 4-8 experimental wells per condition.

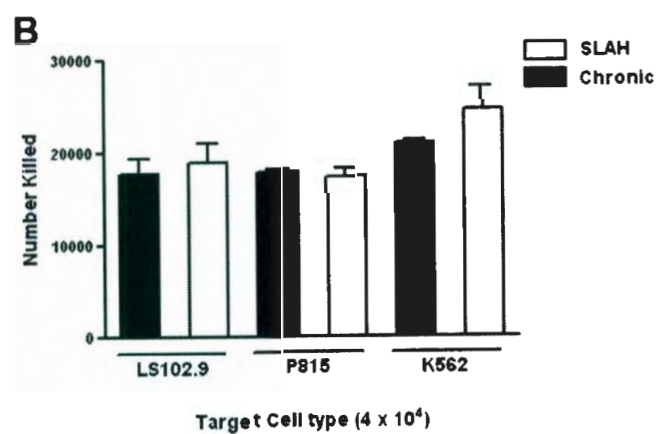
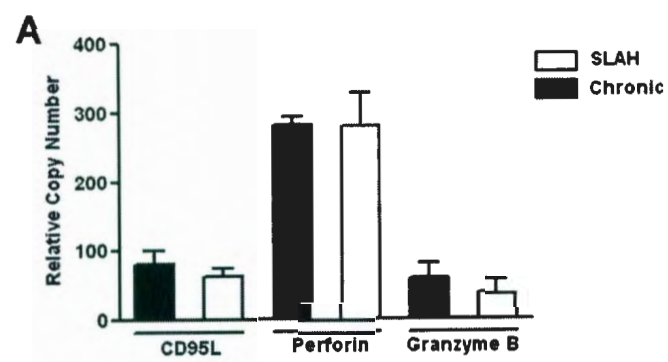
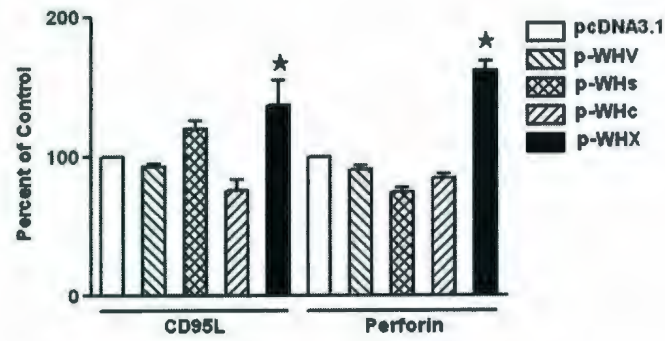
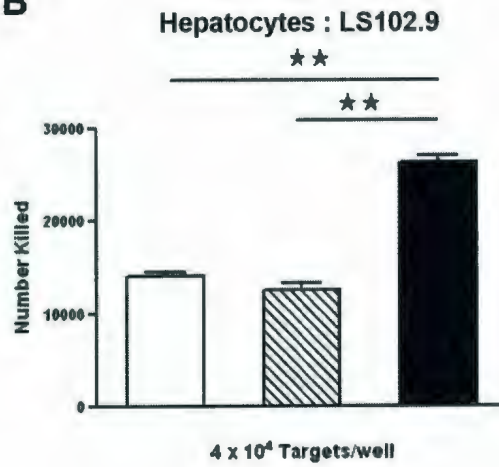
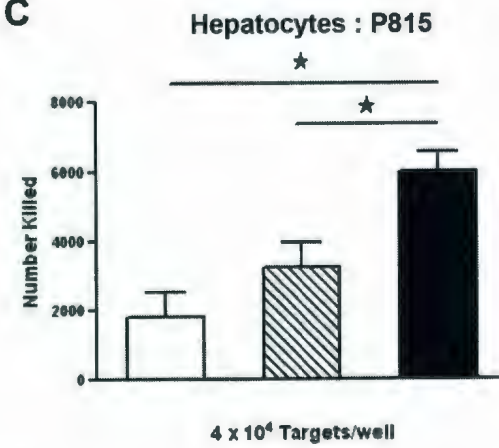
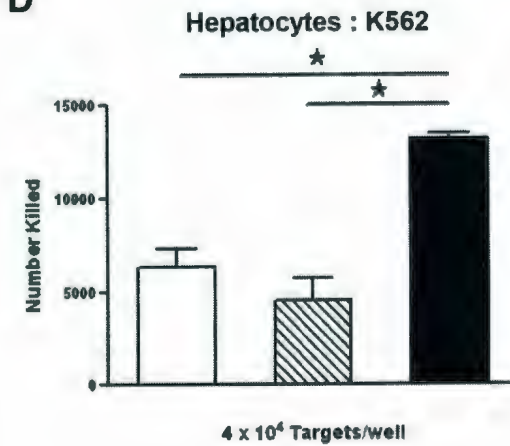


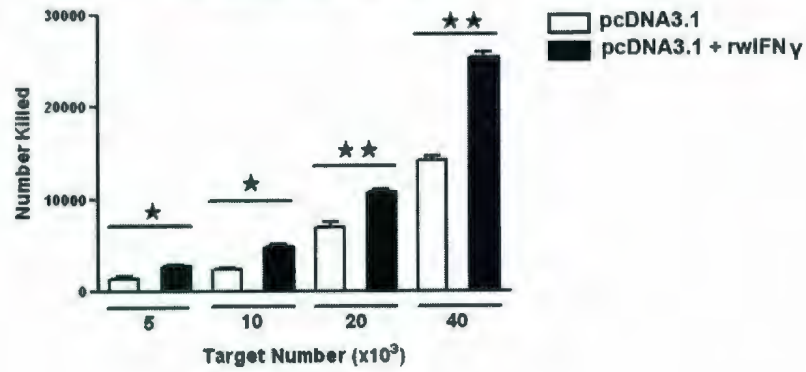
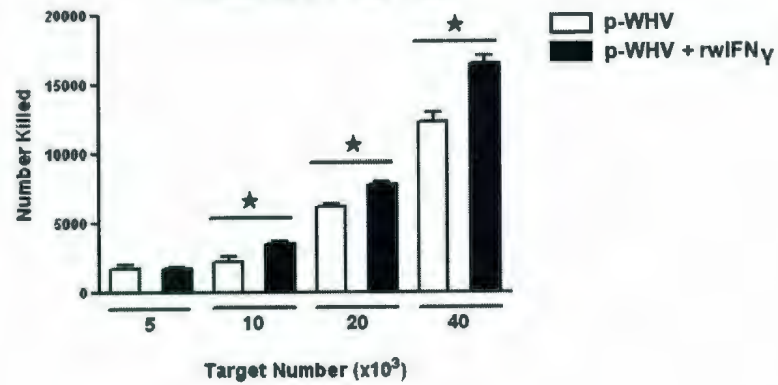
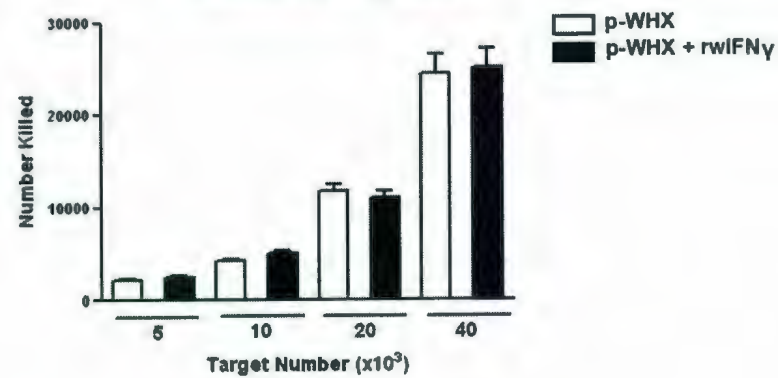
Fig. 4.4. Expression of the cytotoxicity-affiliated genes and the cell killing capacity of woodchuck hepatocytes transfected with complete WHV genome or WHV subgenomic sequences encoding individual viral proteins. WCM-260 hepatocytes stably transfected with empty pcDNA3.1 vector (control) or with p-WHV, p-WHs, p-WHc or p-WHx were analyzed for expression of CD95L and perforin by real-time RT-PCR (A). Data were normalized against β -actin expression and are presented as mean \pm SEM relative to control cells transfected with empty pcDNA3.1 vector which were assigned the value of 100%. The cytotoxic activity of hepatocytes transfected with pcDNA3.1 alone, p-WHV and p-WHx was evaluated by JAM assays against CD95-bearing LS102.9 (B) and P815 (C), and CD95-deficient K562 (D) cell targets, with each assay performed in triplicate with 4-8 experimental wells per condition. Differences in expression of the cytotoxicity-affiliated genes (A) or in the cytotoxic activity (B-D) after hepatocyte stable transfection with complete WHV genome or individual WHV genes were significant in comparison with control cells transfected with empty vector for * at $P < 0.01$ and for ** at $P < 0.001$.

A**B****C****D**

perforin mRNA levels in hepatocytes (Fig. 4.4A). In agreement with these findings, transfection with complete WHV genome or empty vector did not modulate hepatocyte killing of CD95-bearing LS102.9 (Fig. 4.4B) or P815 (Fig. 4.4C) cells, while transfection with p-WHx significantly ($P<0.001$ or $P<0.01$) enhanced elimination of both these cell targets (Figs. 4.4B and 4.4C). Furthermore, perforin-dependent hepatocyte-mediated killing of CD95-deficient K562 cells also was meaningfully ($P<0.01$) augmented following transfection of hepatocytes with p-WHx but not with the complete WHV DNA or empty pcDNA3.1 vector (Fig. 4.4D)

4.3.5 IFN γ augments CD95L-dependent cell killing by hepatocytes transcribing complete WHV DNA but not WHV X gene alone

To ascertain whether hepatocytes expressing complete WHV genome retain their cytotoxic competence and whether the cell killing displayed by hepatocytes transfected with p-WHx can be further enhanced, relevant WCM-260 cell lines and control WCM-260 hepatocytes were treated with rwIFN γ and their cytotoxic activity measured against CD95-bearing LS102.9 targets. The results showed that while rwIFN γ stimulation of hepatocytes transfected with empty pcDNA3.1 vector (Fig. 4.5A) or with complete WHV DNA (Fig. 4.5B) significantly ($P<0.001$ or $P<0.05$) enhanced their cytotoxic capacity, the same treatment was unable to increase the number of cells killed by hepatocytes transfected with p-WHx (Fig. 4.5C). This raises a possibility that hepatocytes expressing the X gene alone achieved their maximum cytotoxic potency prior to exposure to the cytokine and, thus, their killing capacity can not be further augmented by IFN γ .

A**Hepatocytes : LS102.9****B****Hepatocytes : LS102.9****C****Hepatocytes : LS102.9**

4.4 DISCUSSION

Liver inflammation caused by hepadnaviral infection is associated with elimination of hepatocytes and other cells of the liver parenchyma, and the degree of hepatic tissue injury may vary significantly and range from massive to minimal. It has been generally accepted that the induction of hepatitis is largely a consequence of activation of virus-specific CTL and bystander recruitment of other inflammatory cells, including leukocytes and macrophages (Sitia et al., 2004; Sitia et al., 2002; Thimme et al., 2003). Nonetheless, the degree to which these cells directly or indirectly contribute to apoptotic or necrotic death of liver cells remains uncertain. Also, it is not clear whether cells other than inflammatory infiltrate contribute to or modify the degree of liver damage during viral hepatitis. Since our previous findings revealed that hepatocytes constitutively express functionally active cytolytic effector molecules CD95L (Guy et al., 2006) and perforin (Guy et al., 2008, in press, see Chapter 3), and can efficiently eliminate cell targets, we investigated in the present study whether hepadnaviral infection can influence hepatocyte cytotoxic potency mediated by these molecules.

The results obtained in the initial phase of the current study confirmed that not only WHV-induced CH but also that resolution of SLAH is accompanied by upregulated hepatic transcription of IFN γ and, to a lesser extent, TNF α which occurred in the context of minimal to moderate inflammatory alterations in the livers of the convalescent animals (Hodgson and Michalak, 2001). Our previous *in vitro* findings (Guy et al., 2006) demonstrated that stimulation with IFN γ or TNF α of hepatocytes increased their cytotoxicity mediated by CD95L. In general, the results of the current

study are consistent with these previous findings and they suggested that an enhanced CD95L-dependent cytotoxic phenotype exhibited by hepatocytes might be a consequence of intrahepatic elevations in IFN γ and TNF α due to active or residual liver inflammation. Although chronic WHV hepatitis was associated with a significantly ($P < 0.05$; Fig. 1) higher hepatic expression of these two cytokines, the level of CD95L transcription did not differ between animals with CH and those recovered from acute hepatitis. This may suggest that even a moderate increase in intrahepatic IFN γ and/or TNF α , as observed after resolution of SLAH, upregulates expression of CD95L in hepatocytes to the highest level. The significance of the augmented CD95L expression is not clear but, hypothetically, it may contribute to removal of other cells, for example, activated lymphocytes, as well as pertain to fratricidal lysis of hepatocytes in inflamed livers. In this regard, CD95 was found to be readily detectable on activated lymphoid cells infiltrating the liver (Luo et al., 1997). During virus-induced liver inflammation, cytokine-mediated upregulation of CD95L may play a role in hepatocyte self-protection and limit immune-mediated hepatic tissue damage. In a similar manner, it has been proposed that expression of CD95L by α -cells in pancreatic islets may temporarily protect juxtaposed β -cells from initial lysis mediated by small numbers of infiltrating β -cell-specific T cells (Signore et al., 1998). However, severe pancreatic infiltrates finally overcome the protection afforded by α -cell expression of CD95L and induce β -cell death and insulinitis via both CD95L and perforin-dependent mechanisms (Pearl-Yafe et al., 2006). In an analogous manner, sustained and amplified inflammation, which is evident during chronic hepadnaviral infection (Maini et al., 2000), accompanied by augmented CD95L expression by hepatocytes may be insufficient to

limit the extent of liver damage in severe or advanced hepatitis. There also exists a possibility that during chronic hepadnaviral hepatitis, viral proteins may influence the relative susceptibilities of either lymphoid cells or hepatocytes to elimination via cytotoxic mechanisms (Michalak et al., 2000; Nakamoto et al., 1997).

Interestingly, resolution of acute WHV hepatitis was also associated with upregulated CD95 expression by hepatocytes. It has been suggested that hepatocyte expression of CD95L and CD95 may not be tightly correlated with each other (Luo et al., 1997), leading to potential pathogenic effects occurring via a *trans* mechanism wherein CD95L-positive hepatocytes may induce lysis of neighboring hepatocytes which are CD95-expressing. In spite of the classical view that the CD95L-CD95 interaction results in cell death, recent evidence implies a dual role for the CD95L-CD95 interaction-triggered signaling, wherein CD95L expression results in autoprofiferation of CD95-positive cells (Siegel et al., 2000), possibly as a result of cellular activation following reverse signaling through its cytoplasmic tail (Suzuki and Fink, 1998; Suzuki and Fink, 2000). Taken together, the CD95L-CD95 interaction may alternatively be utilized by hepatocytes to promote regeneration. In this regard, liver regeneration following partial hepatectomy has been shown to be impaired in CD95-deficient mice (Desbarats and Newell, 2000). However, it remains undetermined whether hepatocytes required engagement of CD95 or whether regeneration occurred as a result of CD95-mediated signaling and activation of other cells.

Despite increased expression of hepatocellular CD95L in response to IFN γ and TNF α (Guy et al., 2006), constitutive transcription of perforin is not upregulated in

hepatocytes by these cytokines (Guy et al., 2008, in press, see Chapter 3). Nonetheless, the current study demonstrated that hepadnaviral infection invariably leads to an increased usage of the exocytic pathway by hepatocytes to kill target cells. The data from infection with LCMV in perforin-deficient transgenic mice suggest that the perforin pathway is fundamental to control the expansion of CTL during LCMV infection and its absence is associated with greater hepatic infiltrations constituted by both CD8+ and CD4+ T cells (Matloubian et al., 1999). Thus, the perforin pathway may represent an alternate mechanism by which hepatocytes may regulate lymphocyte trafficking through and their infiltration into the liver. It is interesting to note that while recovery from self-limited acute hepadnaviral infection was accompanied by a significant increase in hepatocyte expression of perforin and enhancement of hepatocyte-mediated lysis of CD95-deficient target cells, the levels of granzyme B mRNA were comparable in hepatocytes derived from healthy animals or from those which spontaneously resolved acute WHV infection (Figures 1 and 2). The apparent discrepancy between increased perforin but not granzyme B expression is not uncommon. Thus, it has been noted that CTL infiltrating the livers in patients chronically infected with HCV may differentially express perforin or granzyme B (Pham et al., 2003). Although the mechanisms underlying discordant display of perforin and granzyme B are not completely understood, transcriptional regulators are known to differentially influence perforin and granzyme B expression (Mori et al., 1998; Salcedo et al., 1993). Furthermore, co-expression of these effector molecules is not absolutely required, since perforin has been demonstrated to mediate granzyme-B-independent cytotoxicity (Shresta et al., 1997). Although investigations determining

transcriptional regulation of perforin and granzyme B expression, and the identification of perforin-dependent, granzyme-B independent cytotoxicity have been investigated in lymphoid effector cells, similar regulatory or effector pathways could also be operational in hepatocytes.

HCV has been demonstrated to influence hepatocyte expression of CD95L via a direct effect of the virus nucleocapsid protein (Iken et al., 2006; Ruggieri et al., 2003). Since primary woodchuck hepatocytes isolated after resolution of acute WHV hepatitis demonstrated increased expression and usage of CD95L as well as perforin in comparison with healthy controls, we aimed to determine whether the observed differences were due to a direct viral influence. The expression of the HBV X gene has been shown to upregulate CD95L mRNA and protein in hepatocytes (Lee et al., 2002; Lin et al., 2005a), a finding which we have confirmed in the current study by examining consequences of the WHV X gene expression on hepatocyte transcription of the cytotoxicity affiliated genes. This effect may be indirect, since human HepG2 cells transfected with the HBV X gene have been shown to produce TNF α (Lara-Pezzi et al., 1998b), which we have found to augment expression of CD95L in cultured woodchuck hepatocytes (Guy et al., 2006). The HBV X gene has also been shown to induce secretion of interleukin-18 (IL-18) resulting in autocrine or paracrine upregulation of CD95L in hepatoma cells (Lee et al., 2002). Demonstration in our study of upregulated transcription of perforin in hepatocytes transfected with WHV X gene is a novel finding which adds a further dimension to already well documented transactivating capacity of hepadnaviral X proteins (reviewed in (Murakami, 2001). On other hand, the finding that expression of a complete WHV genome does not upregulate CD95L or

perforin transcription and hepatocyte cytotoxicity may appear contradictory. However, as we have recently documented (Wang and Michalak, 2006) expression of either the complete WHV genome or the WHV X gene alone, may have completely opposite effects. Thus, by studying presentation of MHC class I heavy chain on woodchuck hepatocytes, we have found, similarly as observed in the current study, that while WHV X gene expression significantly augmented the display of MHC class I antigen, expression of complete WHV genome as well as the virus genes encoding for envelope and core proteins was without any measurable effect.

The present study is the first to demonstrate that hepadnaviral infection influences the cytotoxic phenotype of hepatocytes and that this happens irrespective of whether liver disease induced is active or quiescently progresses following resolution of acute hepatitis. The experimental evidence accumulated in our study implies that the increased cytotoxic capability of hepatocytes observed in the course of hepadnaviral infection is a result of virus-induced liver inflammation and predominantly, although unlikely exclusively, is induced and maintained by augmented intrahepatic production of IFN γ and TNF α . The results obtained seem to negate a possibility that the augmented hepatocyte cytotoxic potency could be a direct consequence of virus replication advancing in these cells, although a role for defective virus propagation associated with over-expression of virus X protein can not be entirely excluded. The study provides further evidence that hepatocytes are cytotoxic effectors and can be active contributors to the variety of physiological and pathological processes engaging the liver, including the development and progression of hepadnaviral hepatitis.

CHAPTER FIVE: INTRAHEPATIC INNATE AND ADAPTIVE IMMUNE ACTIVITY IMMEDIATELY AFTER VIRUS INVASION AND IN THE PRE- ACUTE AND ACUTE PHASES OF HEPADNAVIRAL INFECTION

5.0 SUMMARY

Previous studies of HBV-infected patients, in experimentally infected chimpanzees and in HBV transgenic mice have demonstrated the importance of broad anti-viral innate and adoptive responses in inhibition of hepadnaviral replication and recovery from hepatitis. However, there are no systematic studies or conclusive delineations of virological and immunological events occurring immediately after hepadnavirus invasion and during the pre-acute phase of hepadnaviral infection. These very early events might be of primary importance in determining the prolonged incubation period normally observed in hepatitis B and the intrinsic propensity of hepadnavirus to persist. The aim of the current study conducted in the woodchuck model of self-limited acute hepatitis B was to determine the intrahepatic kinetics of hepadnavirus replication and activation of cell subsets of the innate and adoptive immune responses, cytokines and the immune cell effector molecules in sequential liver biopsy samples collected from one hour post-infection forward by quantifying expression of relevant genes by sensitive assays based on real-time polymerase chain reaction. The results revealed that despite a prolonged asymptomatic period preceding the appearance of hepatitis, low-level virus replication is established in the liver as early as one hour post-infection. Further, virus invasion leads within 3 – 6 hours to upregulated hepatic transcription of genes indicative of activation of antigen presenting

cells and NK cells, including significant augmentations in the intrahepatic levels of IFN γ and IL-12 mRNA. Subsequently, NKT cell activation became evident at 48 – 72 hours post-infection. This phase was associated with a significant reduction in the hepatic virus replication, suggesting that this early innate response was, at least partially, successful in limiting virus propagation. Nonetheless, this early response was not followed by T cell activation and liver infiltration until 4-5 weeks later when hepatitis became evident. Collectively, the results obtained imply that hepadnavirus replication is established in the liver almost immediately following exposure to a large, liver pathogenic dose and that the local anti-viral innate response is promptly activated. However, this initial response appears to be insufficient in prompting an effective adaptive virus-specific T cell response capable of early virus elimination, as is the case in the majority of self-limiting infections caused by other viral pathogens.

5.1 INTRODUCTION

HBV is the prototypic member of the *Hepadnaviridae* family of small, enveloped, primarily hepatotropic DNA viruses which cause acute and chronic hepatitis and hepatocellular carcinoma (reviewed in Chisari and Ferrari, 1995). Hepadnaviruses display highly restricted host specificity. Thus, HBV infection is limited to humans and higher primates, while WHV, despite sharing significant structural, genomic and antigenic similarities with HBV, infects the eastern North American woodchuck (*Marmota monax*) (Michalak, 1998). Notwithstanding their limited host ranges, HBV and WHV induce similar courses of liver disease which are preceded by a long incubation period ranging from 6 weeks to 6 months for hepatitis B

and from 4 to 10 weeks for WHV hepatitis (Chisari and Ferrari, 1995; Menne and Cote, 2007). In contrast with viraemia and the appearance of adaptive T cell immunity in a few days after invasion with most viral pathogens, significant increases in HBV replication are not observed until 3-5 weeks p.i. in chimpanzees, while activation of HBV-specific adaptive immunity occurs several weeks later (Bertoletti and Ferrari, 2003; Guidotti et al., 1999; Thimme et al., 2003).

Despite the delay in activation of HBV-specific T cell responses, a robust, multispecific T cell reactivity seems to be essential for both induction of AH and the significant reduction in virus load and clinical recovery (Penna et al., 1996; Penna et al., 1997), although it is unable to entirely clear the virus (Michalak et al., 1994; Rehmann et al., 1996; Rehmann et al., 1995a). Experimental evidence acquired from the woodchuck model of hepatitis B, as well as the chimpanzee and HBV-transgenic mouse systems, have clearly indicated the importance of hepadnavirus-specific CD4⁺ and CD8⁺ T lymphocytes and antiviral cytokines, such as IFN α/β , IFN γ and TNF α , in the downregulation of virus replication (Hodgson and Michalak, 2001; Menne et al., 1998; Menne et al., 2002; Nakamura et al., 2001). While bystander recruitment of nonvirus-specific T cells and other immune subsets greatly contribute to liver inflammation and hepatocyte destruction (Maini et al., 2000; Sitia et al., 2002), the role of antiviral cytokines, particularly IFN γ , has been ascribed to the ability to noncytopathically inhibit hepadnavirus replication (Guidotti et al., 1994; Guidotti et al., 1999). Elevations in the intrahepatic expression of IFN γ appear sufficient in chimpanzees to reduce HBV viraemia before the peak onset of virus-induced hepatitis, as evidenced by augmented serum alanine aminotransferase (ALT; (Guidotti et al.,

1999)). Also, an increased intrahepatic expression of IFN γ is consistently detected for many years following recovery from WHV-induced AH, implying its role in control of residual (occult) WHV replication continuing after resolution of AH (Hodgson and Michalak, 2001).

NK and NKT cells are components of the innate immune system. These cells are enriched among the resident lymphomononuclear cells in the liver, which are capable of producing IFN γ and other cytokines within minutes following viral invasion (reviewed in (Biron et al., 1999)). The results from studies in HBV transgenic mice suggest that artificial activation of NKT cells via the CD1d-restricted glycolipid α -galcer can downregulate viral replication (Kakimi et al., 2000; Kakimi et al., 2001), while non-classical NKT cells may recognize HBV antigens expressed in the liver (Baron et al., 2002). It has been suggested that NK or NKT-derived IFN γ may be the principal mediator of HBV-downregulation in acutely infected chimpanzees (Guidotti et al., 1999), while evidence from the woodchuck model suggests that activated, cytotoxic NK or NKT cells may contribute to hepatocyte killing and recovery from WHV infection (Hodgson et al., 1999).

Nonetheless, despite findings suggesting that hepadnaviruses may directly activate intrahepatic immune cells capable of producing cytokines promoting antiviral defense and favoring a T_H1-type T lymphocyte response (Guidotti et al., 1999; Thimme et al., 2003), comprehensive data from a natural model of hepadnaviral infection are lacking. Furthermore, characterization of the liver immune response immediately following hepadnavirus invasion has not yet been accomplished. The aim of the current study was to recognize, using the woodchuck model of HBV infection, the

nature and the kinetics of intrahepatic immune responses occurring immediately after exposure to WHV and during the pre-acute and acute phases of hepadnaviral infection by quantifying hepatic transcriptional activity of genes encoding for proinflammatory and antiviral cytokines and for markers specifying individual immune cell subsets.

5.2 MATERIALS AND METHODS

5.2.1 Study design

Prior to the study, liver biopsies (LBx 1; see Fig. 5.1) were obtained from 29 young adult healthy, WHV-naïve woodchucks and their fragments were cryopreserved for subsequent DNA and RNA isolations, and fixed in formalin for histological examination. All woodchucks were intravenously infected with the same serum-derived WHV/tm3 inoculum at a dose of 1.1×10^{10} DNase-protected vge (AY334075; (Coffin et al., 2004) and they were randomly subdivided into groups, each comprised of at least two animals (see Table 5.1). Beginning at one hour post WHV injection, liver biopsies (LBx 2) were collected according to the scheme shown in Figure 5.1. Follow-up liver tissue samples (LBx 3) were obtained approximately 6 weeks thereafter (see Fig. 5.1). Serum and PBMC samples were collected weekly until 10 w.p.i. and then monthly for up to 6 to 36 months until euthanasia. Liver tissue, PBMC and serum samples, including those collected at autopsy, were cryopreserved for analysis.

5.2.2 Serological assays and assessment of liver inflammation

Detection of serum WHsAg and anti-WHc was done using immunoassays reported previously (Coffin et al., 2004; Michalak et al., 2004; Michalak et al., 1999).

Figure 5.1. Schematic representation of the time-line of acquisition of liver tissue samples from a cohort of WHV-infected woodchucks investigated in the current study. Liver biopsies (LBx 1) were obtained from each healthy, WHV-naïve animal (n = 29) prior to inoculation with WHV. The animals were then blindly randomized, injected with a liver pathogenic dose of WHV containing 1.1×10^{10} vge, and the second liver biopsy (LBx 2) was taken beginning from one hour post-inoculation (p.i.) as shown in the scheme. The third liver biopsy (LBx 3) was collected at approximately 6 weeks p.i. The animals (n = 29) were followed for 6 to 36 months prior to autopsy.

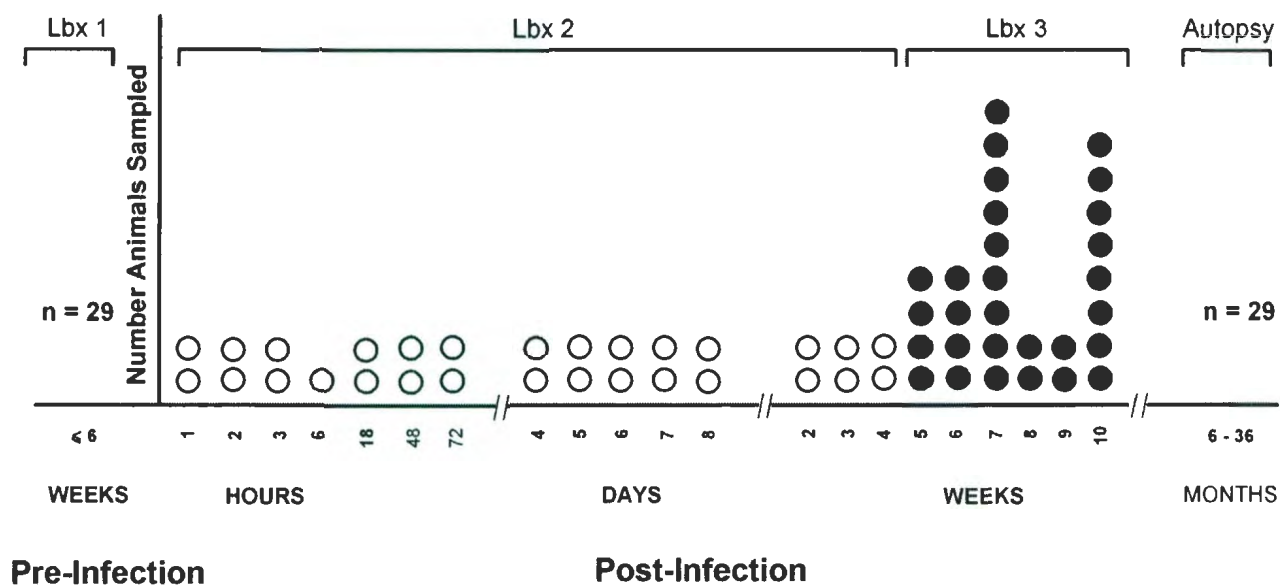


Table 5.1. Experimental groups according to the time elapsing between inoculation
with WHV and acquisition of liver biopsy sample

Group/Time period post-WHV inoculation	Number of liver biopsies	Liver biopsy sequential number
0	29	1
1 – 3 hours	5	2
3 – 6 hours	2	2
18 – 48 hours	3	2
48 – 72 hours	3	2
4 – 8 days	10	2
2 week	2	2
3 week	2	2
4 week	2	2
5 week	4	3
6 week	4	3
7 week	9	3
8 week	2	3
9 week	2	3
10 week	8	3
>6 months	29	4

Histological examination of paraffin-embedded liver tissue fragments was performed following routine processing and staining with hematoxylin and eosin, as reported (Hodgson and Michalak, 2001). Morphological assessment of hepatocellular, extrahepatocellular intralobular and periportal lesions was graded applying previously described criteria (Hodgson and Michalak, 2001; Michalak and Lin, 1994; Michalak et al., 1990).

5.2.3 Nucleic acid isolations

Total DNA was extracted from 250- μ l serum samples or approximately 50 mg of liver tissue using proteinase K digestion, phenol-chloroform-isoamyl alcohol method, as reported (Michalak et al., 2004; Michalak et al., 1999). Total RNA was isolated from liver biopsy samples using Trizol reagent (Invitrogen, Carlsbad, CA), as per the manufacturer's instruction. RNA was treated with DNase to remove potentially contaminating DNA (RNase-free, DNase digestion kit; Sigma, Oakville, ON, Canada) prior to reverse transcription to cDNA, as described elsewhere (Guy et al., 2006).

5.2.4 Detection of WHV DNA and RNA by real-time PCR or RT-PCR

Quantitative assessment of WHV DNA load was done by real-time PCR analysis using 2 μ l of serum-derived DNA or 50 ng of liver biopsy DNA and the Roche lightcycler (Roche Diagnostics, Laval, PQ, Canada). WHV DNA amplicons were generated using the forward primer 5'-ATGCACCCATTCTCTCGAC and reverse primer 5'-CTGAGCAGCTTGGTTAGAGT, yielding a 221-bp fragment which was detected by SYBR green I incorporation. Copy numbers of WHV DNA were

calculated by extrapolation from a standard curve which was generated using 10-fold serial dilutions of plasmid containing known copy numbers of WHV DNA. WHV RNA was similarly detected using 2 µl of cDNA or equivalent to 50 ng total RNA. The specificity of real-time PCR products was confirmed by nucleic acid hybridization (NAH), *i.e.*, Southern blot hybridization analysis, using WHV-specific radiolabelled probes and autoradiography, as reported (Coffin et al., 2004; Michalak et al., 2004). The sensitivity of the real-time PCR assay was <200 vge/µg DNA or RNA, or <50 vge/ml serum.

5.2.5 Detection of WHV cccDNA

WHV cccDNA, representing virus genome replicative intermediate, was detected in liver biopsy samples by PCR amplification (sensitivity of 10^2 vge/µg), as previously described (Lew and Michalak, 2001; Michalak et al., 2004). Briefly, 4 µg of DNA was digested with mung bean nuclease prior to PCR amplification with oligonucleotide primers spanning the nicked region of the WHV DNA genome. PCR amplicons specificity was routinely confirmed by NAH, as reported (Coffin et al., 2004; Michalak et al., 2004).

5.2.6 Identification of woodchuck cellular gene sequences

To facilitate analysis of the spectrum and the dynamics of intrahepatic immune response to WHV infection, a number of woodchuck gene sequences encoding different immune cell specific markers, immune cell effector molecules and cytokines were determined applying a strategy previously reported (Guy et al., 2006). In general,

woodchuck gene sequences were identified by RT-PCR using degenerate primers which sequences were deduced through interspecies comparison of the sequences available in Genbank. The resulting amplicons were cloned into the PCR II TOPO TA cloning system (Invitrogen) and then the excised fragments sequenced in both directions. Table 5.2 presents the list of woodchuck genes for which hepatic expression was quantified in the current study and the primer pairs used for their amplification.

5.2.7 Analysis of woodchuck gene expression by real-time RT-PCR

Real-time RT-PCR assays were developed for each cellular, effector molecule and cytokine gene analyzed, using the Roche lightcycler and SYBR green I detection and PCR primer pairs given in Table 2. Changes in gene expression levels were determined by comparison to the baseline level of each gene transcription detected in the liver biopsy obtained prior to infection with WHV for each individual animal after normalization to expression of the housekeeping gene β -actin. Following measurement of a given gene expression in a particular liver sample, the mean expression level was determined for all liver biopsy samples within the experimental groups, as shown in Table 5.1.

5.2.8 Inactivation of WHV serum pool

To ascertain that the observed changes in the intrahepatic gene expression levels were truly related to infection with WHV but not to injection with serum components present in the WHV inoculum, a control experiment was performed. Thus,

Table 5.2. Primer sequences used in the current study for real-time RT-PCR quantifications of expression of woodchuck genes encoding immune cell markers, cytokines and cytotoxicity effector molecules

Gene	Primer sequence	Expected size of amplicon (bp)	GenBank accession number
CD3	F-ctgggactctgcctcttattc R-gctgccctttccggatgggctc	536	AF232727
CD4*	F-ggagaataagaagatagagg R-tcaagagtcacagtcagg	560	EF621765
CD8*	F-aacgagggtactatttctgctc R-gtttccgggtgacagatga	309	EF621766
CD40 Ligand*	F-agcatgtgtgtacagt R-ccgccctgagtaagatt	214	EF621770
CD1d*	F-tcctagattagggaagtcagaac R-gctcggagataccacg	222	EF621767
NKp46*	F-ttgccacctagtgcag R-caccaggagcatcacc	200	EF621768
CD95 Ligand	F-ccatttaacagtaagccc R-tcatcatcttgcctcc	250	AF152368
Perforin	F-gcatcaacaatgactggcggg R-tgaagtgggtgccgtagtgtgg	302	AF298158
IL-4	F-tttgctgtccccaagaac R-cctggattcactcacgg	200	AF333965
IL-8*	F-ggtaacctgcctactttc R-gttcaggcaaagctct	207	EF216348
IL12p40	F-ttggattggcaccctgacac R-gcatctggctcagaacttcac	198	X97019
IFN γ	F-aggagcatggacacatca R-ccgaccccgaatcgaag	215	AF232728
TNF α	F-tgagcactgaaagtatgatcc R-tgctacaacatgggtacag	283	AF333967
2',5'-OAS	F-tcaggcaaaggcactaccc R-acttctcttccggacatgct	150	AF082498

F, forward primer; R, reverse primer.

* Woodchuck gene sequence identified for the purpose of the current study.

WHV/tm3 infectious inoculum was inactivated by treatment with 50 µg/ml psoralen (Sigma) combined with exposure to 365 nm UV for 90 min at 4⁰ C. Animals were injected intravenously with 0.5 ml of psoralen-inactivated serum, containing equivalent to 1.1×10^{10} vge, or with 0.5 ml of similarly treated healthy woodchuck serum. The animals were euthanized 3 days post injection.

5.2.9 Accession numbers of woodchuck gene sequences identified

The accession numbers for the woodchuck gene fragment sequences established in the course of the current study and submitted to GenBank were as follows: CD4 gene, EF621765; CD8 gene, EF621766; CD40 Ligand (CD40L), EF621170; CD1d, EF621767; NKp46, EF621768, and interleukin-8 (IL-8), EF-126348.

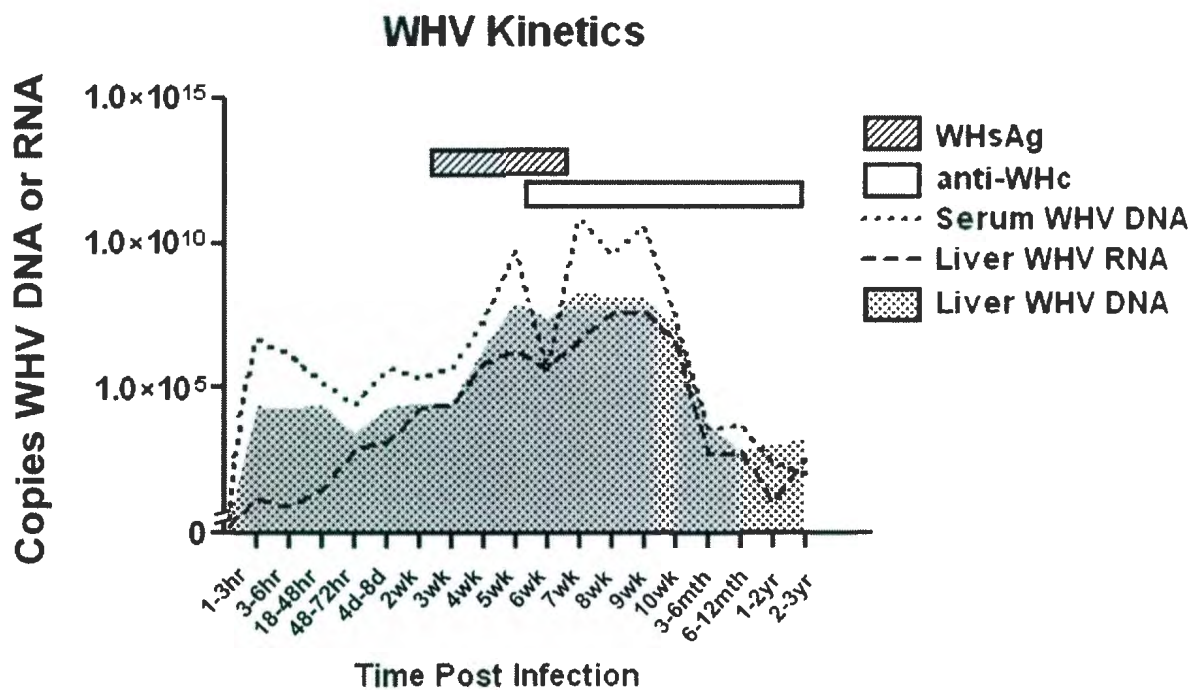
5.3 RESULTS

5.3.1 Serologic and hepatic profiles of WHV infection

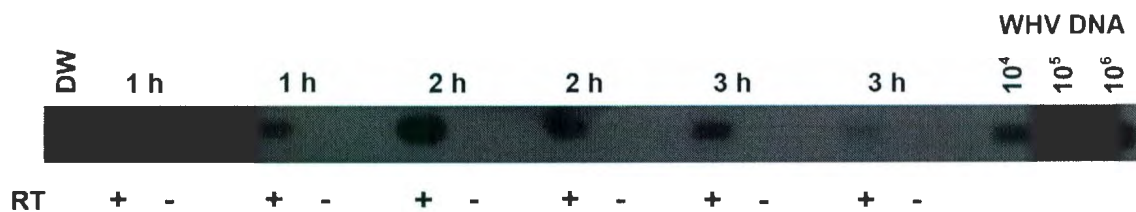
Inoculation of woodchucks with a WHV dose of 1.1×10^{10} vge resulted in transiently serum WHsAg-positive infection in all 29 animals. The mean time of WHsAg appearance was 18.5 days, while its average duration in the circulation was 35.8 days. Anti-WHc appeared on average at 32.6 days after inoculation with WHV and they were detectable until the end of the observation period (Fig. 5.2A). The pattern of serum WHsAg positivity implied, based on the known serological and histological profiles of experimental WHV infection induced with liver pathogenic doses of infectious WHV (Michalak et al., 2004), that a self-limited episode of AH had developed in all woodchucks investigated.

Figure 5.2. Serological and molecular analyses of WHV infection markers following inoculation with a WHV infectious pool containing 1.1×10^{10} vge per dose. (A) Circulating WHV surface antigen (WHsAg) and antibodies to WHV core (nucleocapsid) antigen (anti-WHc) were detected by specific ELISA in serial serum samples collected as described in Materials and Methods. WHV DNA was quantified by real-time PCR in DNA samples extracted from serum or liver biopsy samples. Levels of WHV RNA were determined by real-time RT-PCR using cDNA transcribed from total RNA isolated from liver tissue. WHV DNA or WHV RNA copy numbers are presented as the mean expression levels determined for each experimental group of animals as identified in Table 1. (B) RNA was extracted from liver biopsies obtained at one hour ($n = 2$), 2 hours ($n = 2$) and 3 hours ($n = 2$) post-infection with WHV. RNA samples were treated with DNase prior to transcription (RT+) or not (RT-) to cDNA, as described in Materials and Methods. WHV cDNA equivalents to 50 ng of total RNA were subsequently quantified by real-time PCR and amplicons visualized by agarose gel electrophoresis and Southern blot hybridization analysis using ^{32}P -labeled recombinant WHV DNA as a probe.

A



B



In previous studies, hepadnaviral DNA in the circulation and its replication status in the liver was never assessed in the first few days after exposure to the virus. In the current study, WHV DNA was quantified in serum and liver tissue samples acquired from one hour p.i. forward. Not surprisingly, WHV DNA was detectable in serum at the first time point of examination, *i.e.*, one hour p.i. (Fig. 5.2A). This was due to carry over of WHV genomes from the inoculum which contained a massive amount of virus, *i.e.*, 1.1×10^{10} DNase-protected vge. Subsequently, the serum level of WHV DNA progressively decreased until 48-72 hours p.i. and then slowly increased until a sudden expansion at 3 to 4 weeks p.i. (Fig. 5.2A), culminating in peak detection at week 7 p.i. (a mean level of 3.2×10^{10} vge/ml). The mean WHV DNA level in the circulation showed minor fluctuations during the peak phase of acute WHV infection, occurring between weeks 6 and 9 p.i., prior to a sudden protraction by more than 7 log after week 9 p.i. (Fig. 5.2A). However, in agreement with our previous studies (Hodgson and Michalak, 2001), WHV DNA remained consistently detectable in serum at a mean level of 175 vge/ml during follow-up lasting up to 3 years p.i.

WHV genomes were also detectable in hepatic tissue beginning from one hour after inoculation with virus. WHV DNA load transiently and uniformly in all animals declined slightly and not significantly between 3-6 hours p.i. and significantly ($P = 0.004$) between 48-72 hours p.i. reaching a level approximately 10-fold lower than that detected between one and 6 hours p.i. (Fig. 5.2A). Paralleling WHV DNA detection in serum, the intrahepatic level of WHV DNA exhibited a sudden exponential increase at 3 – 4 weeks p.i. (Fig. 5.2A) prior to reaching the mean peak detection of 4×10^9 vge/ μ g total DNA at 7 weeks p.i. Despite a greater than 4 log reduction in hepatic WHV DNA

load beginning at week 9 p.i., WHV genomes were consistently detectable in the liver for up to 3 years p.i. at a mean level of 2.3×10^4 vge/ μ g total liver DNA.

It remained unclear whether the WHV DNA detected in the liver in the first few hours post-exposure reflected the virus originating from the inoculum, which was passing through or was trapped within hepatic tissue, or from the virus actively replicating in hepatocytes. However, the finding of a transient but significant ($P = 0.004$) lowering in the hepatic load of WHV DNA at 48-72 hours p.i. which was replenished 24 hours later, strongly suggested that the virus was actively replicating, at least from 96 hours p.i. forward.

To directly determine the status of WHV replication in the liver, a PCR/NAH assay specifically detecting WHV cccDNA was employed. The results showed that WHV cccDNA signal was not detectable between one and 6 hours p.i., but was evident in the liver at 18 hours p.i. and forward (data not shown).

By applying real-time RT-PCR detecting WHV RNA, low quantities of WHV transcripts (2.6×10^2 copies per μ g total RNA) were identifiable as early as one hour p.i. (Fig. 5.2A). In contrast with hepatic WHV DNA, which level only slightly increased until 3 – 4 weeks p.i., WHV RNA level showed a steady, prominent augmentation beginning at one hour p.i. and peaking at a mean level of 7.2×10^8 WHV transcripts per μ g total RNA between weeks 8 and 9 p.i. (Fig. 5.2A). A possibility of contamination of cDNA preparations with viral DNA was excluded by DNase treatment of RNA samples prior to RT reaction and by PCR amplification of both transcribed and non-transcribed mRNA samples. As shown in Figure 5.2B, only RNA samples which were reversely transcribed demonstrated WHV cDNA signals, while

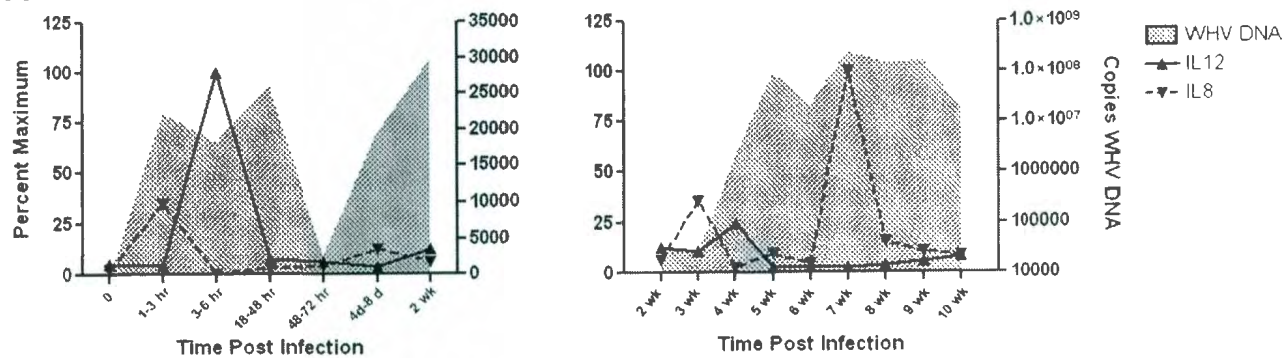
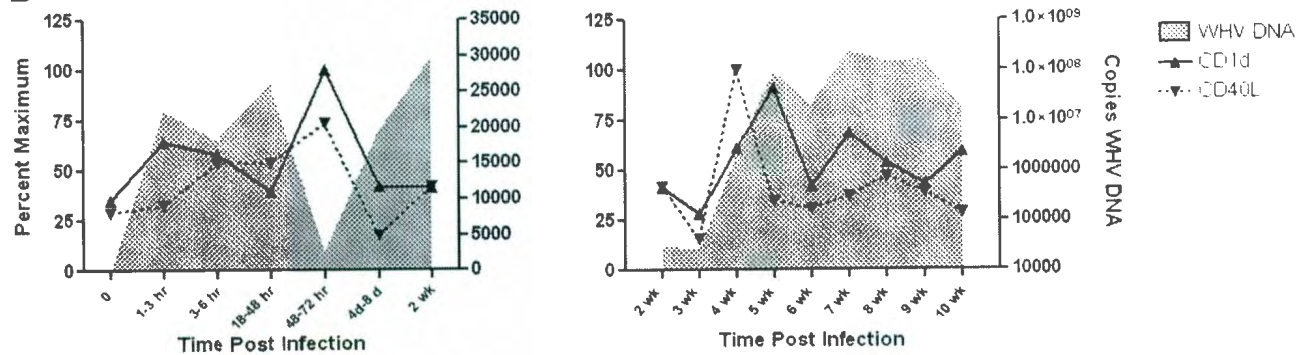
those similarly treated in the absence of reverse transcriptase remained negative even when the amplification products were analyzed by NAH. These results confirmed strict specificity of the WHV RNA detections. Taken together, the results showed that infection with a massive, liver pathogenic dose of WHV results in an immediate, although low-level infection of the liver as evidenced by detection of viral transcripts as early as one hour p.i.

5.3.2 WHV invasion promptly activates antigen presenting cells in the liver

Since injection with WHV resulted in the initiation of virus replication in the liver as early as one hour p.i., it was of interest to determine whether the virus may activate APC, which should be the first cell type recognizing viral intrusion. In this regard, the liver expression of IL-12, a key cytokine produced by APC involved in innate immune responses (Ma and Trinchieri, 2001), IL-8, a strong chemoattractant mediating chemotaxis of phagocytic cells (Kobayashi, 2008), CD1d, a key molecule facilitating antigen presentation by APC to NKT cells (Baron et al., 2002; Kronenberg and Gapin, 2002), and CD40L involved in activation of APC via CD40-CD40L, were quantified by real-time RT-PCR assays specifically developed for the purpose of this study.

Figure 5.3 shows that IL-12 achieved a maximum hepatic expression (~20-fold increase over the pre-infection level; $P = 0.02$) between 3 and 6 hours p.i. (Fig. 5.3A, left panel). In contrast with this early increase, the remaining course of WHV infection, including the period of histologically evident AH with peaking liver injury

Figure 5.3. WHV infection upregulates intrahepatic genes indicative of activation of antigen presenting cells. (A) Expression of IL-12 and IL-8. (B) Expression of CD1d and CD40L. mRNA levels were quantified by real-time RT-PCR in liver biopsy samples collected at time points post-infection as outlined in Table 1. Data shown represent the mean expression levels for each group of animals analyzed at the time point indicated and are presented relative to the maximum level detected for each gene analyzed. Mean level of intrahepatic WHV DNA was determined as described in Materials and Methods and in the legend to Figure 2A. WHV DNA profiles are shown for the time periods between 0 and 2 weeks p.i. and between 2 and 10 weeks p.i. is shown for reference in each panel. Abbreviations: h, hour; d, day and wk, weeks post-inoculation with WHV.

A**B**

between weeks 6 and 8 p.i., was without any noticeable increase in expression of this cytokine (Fig. 5.3A, right panel).

The transcription level of IL-8 increased ($P = 0.1$) by 12.5-fold within one to 3 hours p.i. over that detected in the period prior to infection (Fig. 5.3A, left panel). However, the maximum expression of this cytokine (a 35-fold increase over the pre-infection level; $P = 0.05$) occurred at week 7 p.i., during the phase of acute liver inflammation (Fig. 5.3A, right panel).

Since strong induction of IL-12 expression detected at 3-6 hours p.i. could be directly associated with activation of APC (Ma and Trinchieri, 2001), the intrahepatic expression of CD1d, which facilitates antigen presentation to NKT, cells was also evaluated. The results showed that the level of CD1d mRNA reached a peak (a ~3-fold increase over the pre-infection level; $P = 0.03$) by 48 – 72 hours p.i. (Fig. 5.3B, left panel). Then, the level subsided until WHV replication was drastically augmented at 3-4 weeks p.i. (Fig. 5.3B, right panel). Thus, the upregulated expression of CD1d at 48 - 72 hours was associated with a significant one log decrease in the hepatic WHV DNA load. Furthermore, the concomitant increase ($P = 0.02$) in CD40L expression (Fig. 5.3B, left panel) suggested reciprocal activation of APC via CD40-CD40L interaction.

5.3.3 Activation of hepatic NK and NKT cells immediately follows infection with WHV

WHV infection appeared to induce two distinct phases of very early immune activation in the liver, one between 3 - 6 hours and a second at 48 – 72 hours p.i. Since experimental evidence from other viral infections clearly indicate the ability of NK and

NKT cells to respond within minutes or hours to virus by secretion of IFN γ or by acquisition of cytotoxic function (Biron et al., 1999), expression of the gene encoding the NK receptor NKp46 was investigated.

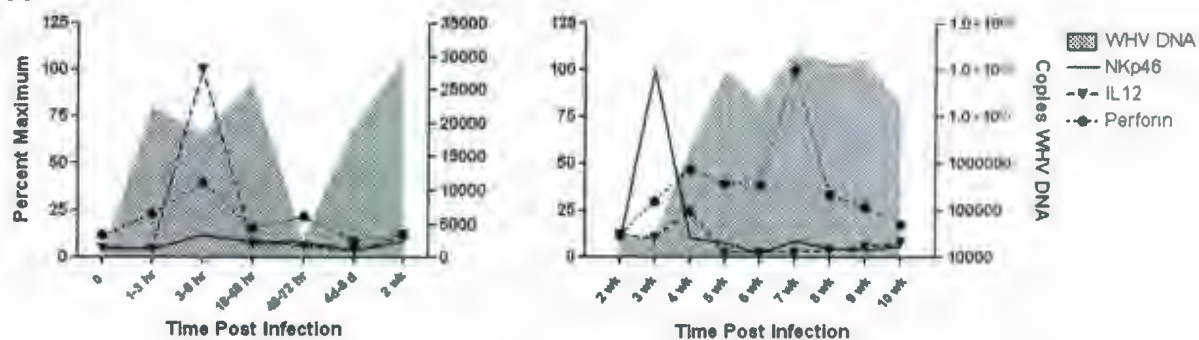
Furthermore, since IL-12 is recognized as a key cytokine which influences IFN γ secretion by NK cells, and also increases NK-mediated cytotoxicity, which is primarily facilitated by the perforin pathway (Moretta et al., 2007), perforin mRNA levels were also quantified. The results showed that the earliest increase in transcription of IL-12, occurring between 3 to 6 hours p.i. (Fig. 5.3A, left panel) coincided with moderately increased expression of NKp46 (a 2.5-fold induction; $P = 0.13$) and perforin (a 3.4-fold induction; $P = 0.13$) (Fig. 5.4A and Fig. 5.4B, left panels, respectively), suggesting activation of NK cells at this very early stage. Furthermore, transcription of IFN γ was also significantly (a 3.3-fold; $P = 0.018$) increased at the same time (Fig. 5.4B, left panel), further supporting possible activation of NK cells.

As it was shown in Figure 5.3B, the early expression of CD1d peaked at 48-72 hours p.i. This finding raised the possibility that APC may, at this stage, display an increased capacity to present antigens to CD1d-restricted NKT cells (Kronenberg and Gapin, 2002). The activation of NKT cells may result in upregulated expressions of IFN γ , as well as IL-4 (Bendelac et al., 2007). As the data obtained indicated, enhanced CD1d expression was associated with relatively slight but noticeable increases in both IFN γ (a 4-fold; $P = 0.032$) and IL-4 (a 2-fold; $P = 0.065$) transcription at 48 – 72 hours p.i. (Fig. 5.4B, left panel).

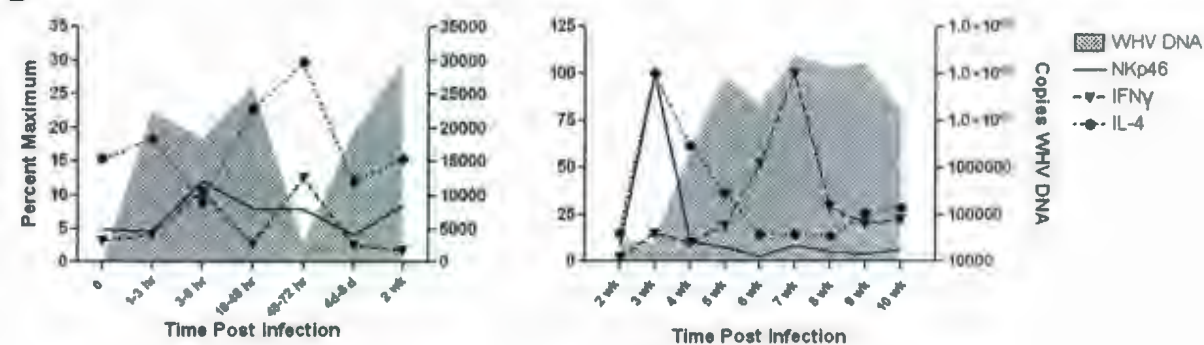
After week 2 p.i., not surprisingly, IFN γ and perforin mRNA levels reached their maximum at week 7 p.i., during the peak of histologically evident liver injury,

Figure 5.4. Expression profiles of hepatic genes indicative of activation of NK and NKT cell subsets in woodchucks inoculated with WHV. Liver biopsy samples were analyzed by real-time RT-PCR for expression of cytokines and activation molecules associated with (A) NK cells, (B) NKT cells and (C) antiviral innate activity. Data shown represent the mean expression levels for each group of animals at the time point indicated, and are presented relative to the maximum level detected for each gene analyzed. Mean detection levels of intrahepatic WHV DNA was determined by real-time PCR analysis and presented as described in the legends to Figures 2A and 3.

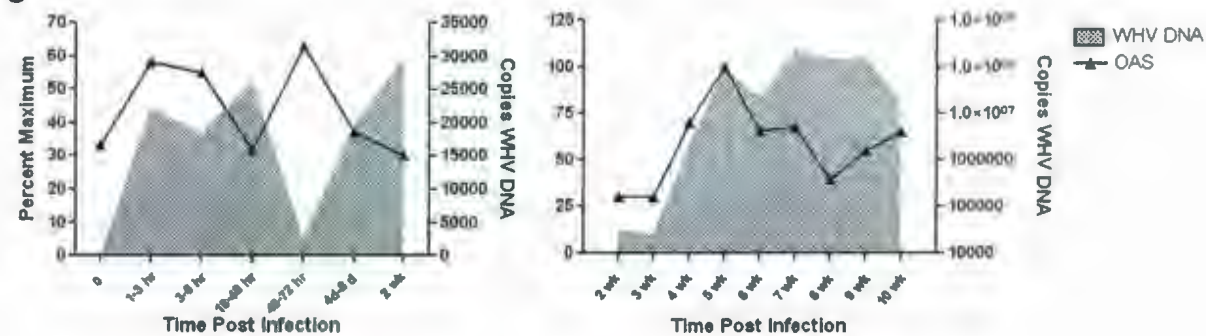
A



B



C



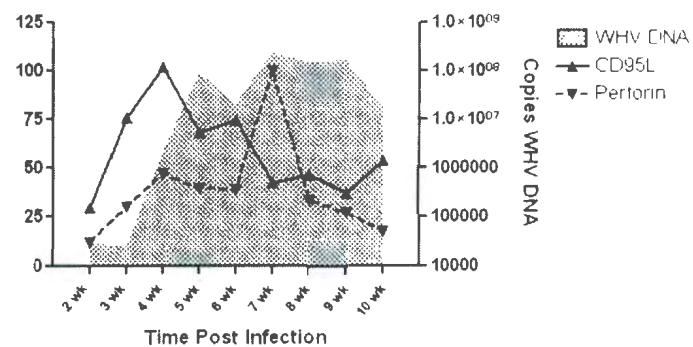
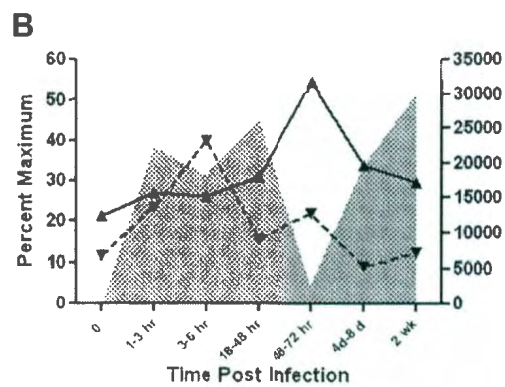
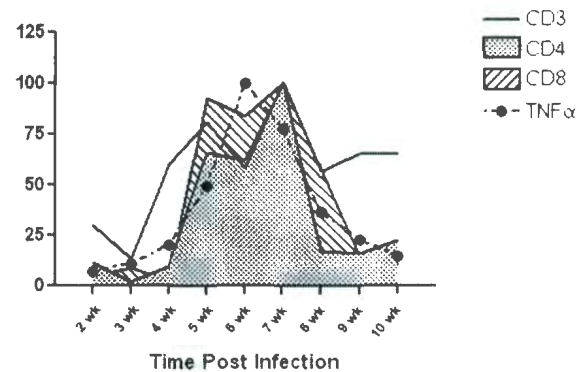
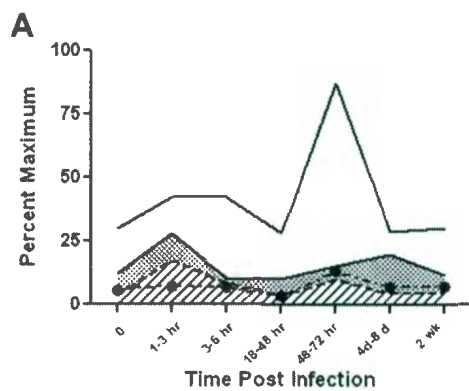
which is normally characterized by liver lymphomononuclear infiltration (see Fig. 5.5A) (Michalak, 1998). Intriguingly, intrahepatic transcription of IL-4 and that of the NK cell marker, NKp46, reached maximum at week 3 p.i., *i.e.*, before viral expansion in the liver (Fig. 5.4B, right panel).

Activation of innate immune effector mechanisms in response to hepadnaviral infection was further suggested by an augmented expression of the anti-viral 2'-5'-OAS (Fig. 5.4C). Thus, detection of WHV DNA and WHV RNA, as early as one to 3 hours p.i., was associated with a slight increase (1.7-fold; $P = 0.14$) in 2'-5'-OAS mRNA level, as determined by real-time RT-PCR (Fig. 5.4C, left panel). Furthermore, a significant increase ($P = 0.05$) in 2'-5'-OAS at 48 – 72 hours p.i. (Fig. 5.4C, left panel) correlated with a significant ($P = 0.03$) elevation in IFN γ expression (Fig. 5.4B, left panel), a known inducer of 2'-5'-OAS (Mullan et al., 2005; Wang et al., 2007). Evaluation of the induction of 2'-5'-OAS in all animals examined at 48 – 72 hours p.i. (including those presented in Figure 5.6; total $n = 7$), a significant increase increase in mRNA expression was observed ($P = 0.02$). This occurred at the time when hepatic WHV load significantly decreased. Taken together, this pattern of events strongly supports the possibility that the transient upregulation of hepatic IFN γ occurring in the first 72 hours following hepadnaviral exposure was biologically relevant and exerted a strong antiviral effect.

5.3.4 Intrahepatic CD4⁺ and CD8⁺ T cells are quiescent for weeks after WHV infection

The prominent reduction in hepadnavirus replication and resolution of AH appear reliant upon a strong and multispecific antiviral T cell response which is characterized by secretion of IFN γ and TNF α (Guidotti et al., 1994; Guidotti and Chisari, 2001). To assess whether the observed initial elevations in intrahepatic expression of IFN γ (Fig. 5.4B, left panel) could be due to the presence of activated T cells, hepatic expression of CD4, CD8 and CD3 T cell markers was quantified. It was found that CD4 and CD8 mRNA levels were slightly elevated (20-25% over the pre-infection levels; $P = 0.18$ and $P = 0.12$, respectively) in the first 3 hours post WHV exposure, then their levels subsided and remained consistently low (10-15% of the pre-infection levels; $P = 0.3$ and $P = 0.2$, respectively) until week 4 p.i. (Fig. 5.5A, left panel). However, not surprisingly, very prominent increases in the mRNA levels of CD4 (a 8.3-fold increase; $P = 0.017$) and CD8 (a 26.7-fold increase; $P = 0.03$) were detected during the peak of histologically evident liver inflammation, which also correlated with detection of the maximal levels of IFN γ mRNA (a 31.3-fold induction; $P = 0.003$) and TNF α mRNA (a 17-fold induction; $P = 0.07$) (Fig. 5.5A, right panel). In contrast to CD4 and CD8, the level of CD3 mRNA showed a distinctive peak (a 2.9-fold increase; $P = 0.03$) at 48 – 72 hours p.i. (Fig. 5.5A, left panel). Subsequently, the CD3 expression level became again augmented beginning from week 3 p.i., preceding by approximately one week the rise in the CD4 and CD8 transcription. Then, the expression profiles of CD3, CD4 and CD8 paralleled each other both during and after acute phase of hepatitis (Fig. 5.5A, right panel). These results suggested that

Figure 5.5. Expression of T cell-affiliated genes in sequential liver samples collected from animals infected with a liver pathogenic dose of WHV. (A) Real-time RT-PCR analyses of CD3, CD4 and CD8 T cell markers and expression of antiviral cytokines IFN γ and TNF α in liver biopsy samples collected as outlined in Figure 1. (B) The levels of the cytotoxic effector molecules CD95L mRNA and perforin mRNA were similarly determined. Data shown represent the mean expression levels for each group of animals at the time point indicated, and are presented relative to the maximum level detected for each gene analyzed. Mean detection levels of intrahepatic WHV DNA was determined and presented as outlined in the legends to Figures 2A and 3.



upregulated intrahepatic expression of IFN γ (Fig. 5.4B, left panel) or TNF α (a 2.2-fold increase over the pre-infection level; $P = 0.08$) (Fig. 5.5A, left panel) detected at 48 – 72 hours p.i. reflected activation of cells of the innate immune system rather than conventional T cell subsets, however a contribution of the latter cannot be completely excluded. The augmented expression of CD3, CD1d (Fig. 5.3B, left panel) and IFN γ (Fig. 5.4B, left panel) at 48 – 72 hours p.i. implied that NKT cells also became activated shortly after exposure to pathogenic hepadnavirus.

In addition, the enhanced expression of CD3, preceding the rise in CD4 and CD8 expression during the acute phase of WHV hepatitis (Fig. 5.5A, right panel), and co-occurring with the augmented transcription of NKp46 may suggest that the activation of both NKp46-positive NK cells and CD3-positive NKT cells was taking place before major infiltration of the liver with CD4 $^{+}$ and CD8 $^{+}$ T lymphocytes (Fig. 5.5A, right panel). In addition, it appears that CD3-positive NKT cells at this relatively late stage of infection, *i.e.*, week 3 p.i., were responding by producing IL-4 (Fig. 5.4B, right panel).

5.3.5 Upregulated transcription of CD95L and perforin correlates with early immune response to WHV

Figure 5.5B (left panel) and Fig. 5.4A (left panel) show that the intrahepatic expression of perforin was moderately elevated at 3 to 6 hours (3.4-fold; $P = 0.13$) and also augmented (1.9-fold; $P = 0.05$) at 48 – 72 hours p.i. To ascertain whether an increase in the liver local cytotoxic activity might be responsible for the transient decreases in hepatic WHV DNA detected at 3-6 hours and 48-72 hours p.i., expression

of CD95L, an effector molecule capable of inducing death of hepatocytes which are naturally endowed with CD95 (Ogasawara et al., 1993), was quantified by real-time RT-PCR. As shown in Figure 5.5B (left panel), CD95L was significantly ($P = 0.03$) upregulated (a 2.6-fold) at 48 – 72 hours p.i., suggesting that indeed augmented hepatic cytotoxicity, possibly mediated by both perforin and CD95L, might contribute to the transient depletion of WHV DNA seen at 48-72 hours p.i. In the time period after week 2 p.i., CD95L expression in the liver became noticeably enhanced, reaching maximum (a 4.8-fold increase; $P = 0.01$) at week 4 p.i. (Fig. 5.5B, right panel). Surprisingly, this CD95L mRNA peak preceded both the expansion of virus in hepatic tissue as well as the rise in CD4 and CD8 mRNA levels coinciding with histologically evident AH (Fig. 5.5A, right panel). On the other hand, this peak of CD95L expression occurred together with the upregulated transcription of CD3, suggesting a possible contribution of NKT cells to the increased intrahepatic detection of CD95L. However, we have previously reported that also hepatocytes constitutively express CD95L (Guy et al., 2006). Thus, it cannot be completely excluded that increased intrahepatic detection of CD95L mRNA was not related to elevated expression by hepatocytes.

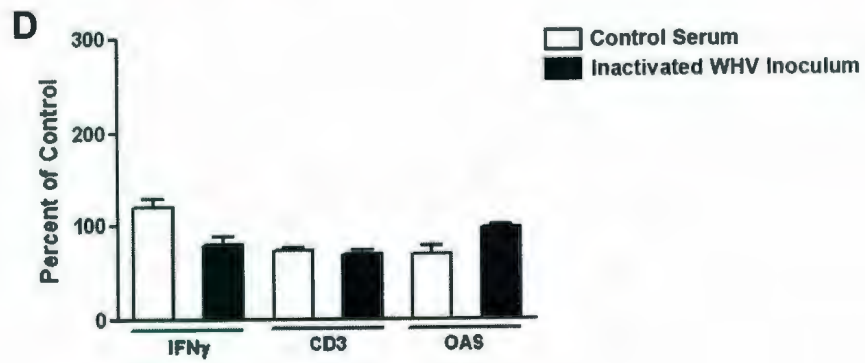
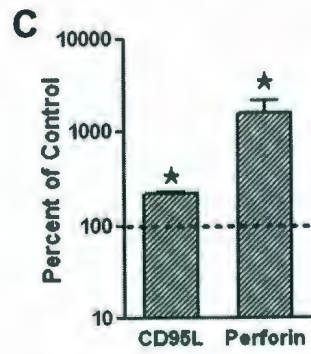
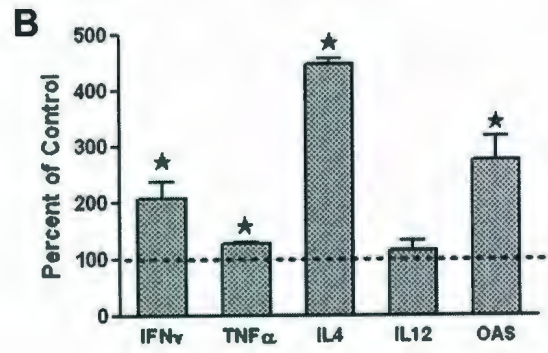
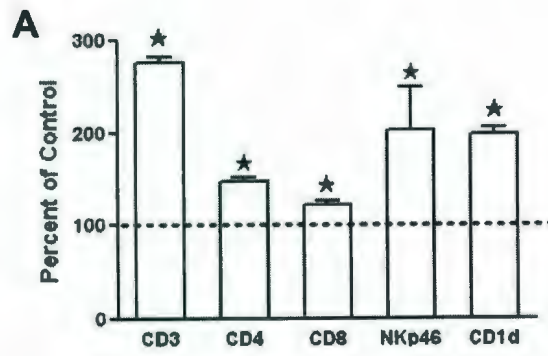
5.3.6 WHV invasion reproducibly induces liver immune activation at 72 hours post-exposure

To confirm that inoculation with a large dose of WHV was associated with activation of intrahepatic immune response as early as 72 hours p.i., two additional healthy woodchucks were intravenously injected with 1.1×10^{10} vge of the WHV/tm3 inoculum and were euthanized at 3 days p.i. In agreement with the first set of our data,

significant elevations in CD3, NKp46 and CD1d expression levels were detected 72 hours after injection with virus, suggesting an accumulation and/or local activation of NK and NKT cells (Fig. 5.6A). However, transcriptions of CD4 and CD8 were also meaningfully upregulated (by 47% and 21%, $P = 0.001$ and $P = 0.003$, respectively) when compared with the hepatic expression of these genes prior to WHV infection (Fig. 5.6A). Despite these increases, we did not notice any morphological evidence of inflammatory infiltrations at this time point, which may suggest that this augmented expression could be due to the activation of the cells already residing within the liver.

Quantification of the cytokine expression levels at 72 hours p.i. also confirmed the first set of data which showed significant elevations of IFN γ and IL-4, possibly reflecting activation of NKT cells (Fig. 5.6B). In addition, a significant ($P < 0.0001$) increase in TNF α may reflect activation of intrahepatic macrophages, as was previously suggested in regard to a transient elevation in IL-12 mRNA (Fig. 5.3A, left panel) at 3 to 6 hours p.i., which had subsided by 72 hours p.i. (Fig. 5.3A, left panel and Fig. 5.6B). Similarly, as shown in Figure 5.4C (left panel), detection of 2'-5'-OAS mRNA was significantly ($P = 0.0017$) elevated at 72 hours (Figure 5.6B), implying that the invading WHV is recognized by effector immune cells. Finally, expression of both cytotoxic effector molecules CD95L ($P < 0.0001$) and perforin ($P = 0.047$) were significantly elevated at 72 hour p.i. (Figure 5.6C), raising the possibility that both noncytopathic as well as cytotoxic mechanisms may be activated in the liver against WHV at this very early stage of infection.

Figure 5.6. Challenge with a WHV inoculum, but not with inactivated hepadnavirus, upregulates genes indicative of intrahepatic immune response activation at 72 hours post-infection. Two adult woodchucks were injected with a large infectious dose of WHV (1×10^{10} vge) prior to euthanasia 72 hours later. Real-time RT-PCR analyses were performed to determine intrahepatic expression of selected markers denoting (A) immune cell subsets, (B) anti-viral cytokines and mediators or (C) cytotoxic effector molecules. Data represent the mean gene expression levels determined for both woodchucks, with each cDNA sample analyzed in triplicate. In control experiments (D), woodchucks were inoculated with 0.5 ml healthy woodchuck serum or with 0.5 ml of the previous serum WHV inoculum, containing 1×10^{10} vge, which had been inactivated by treatment with psoralen and UV light. Animals were sacrificed 72 hours p.i. and intrahepatic mRNA levels of selected genes indicated were quantified by real-time RT-PCR. Gene expression levels shown in panels A - D are presented relative to those determined in healthy liver biopsy samples of the same animals obtained prior to injection with the infectious WHV-serum pool or inoculation with healthy control or inactivated serum.



5.3.7 Infectious WHV is required to activate hepatic immune response

To exclude the possibility that exposure not to WHV but to components of serum carrying the virus might be responsible for activating intrahepatic immune reactivity detected at 72 hours after inoculation, additional control experiments were performed using inactivated inoculum or healthy control serum. For this purpose, a sample of woodchuck serum serving as WHV/tm3 inoculum and containing 1.1×10^{10} WHV vge and serum from a healthy woodchuck were treated with psoralen and exposed to ultraviolet light, as outlined in Materials and Methods. As shown in Figure 5.6D, inactivated WHV inoculum or similarly treated control serum were unable to upregulate expression of the hepatic genes which have been previously found to be significantly augmented at 72 hours post-exposure. Specifically, there were no increases in IFN γ or CD3 mRNA levels and no induction of 2'-5'-OAS (Figure 5.6D). These results confirmed that productive WHV infection, but not exposure to viral antigens or serum components, was responsible for activation of intrahepatic immune responses as depicted in our study.

5.4 DISCUSSION

A unique feature of hepadnaviral hepatitis is a prolonged incubation period where no apparent clinical symptoms or biochemical evidence of liver injury are evident. Previous studies, in both the woodchuck model of hepatitis B (Cote et al., 2000; Menne and Cote, 2007; Menne et al., 1998) and in HBV-infected chimpanzees (Murray et al., 2005; Wieland et al., 2004b), have suggested that viral replication remains largely undetectable until 3 – 4 weeks p.i., at which time exponential virus

expansion leads to infection of all or almost all hepatocytes. These studies have also demonstrated that antiviral immunity, mediated predominantly by virus-specific CD8⁺ CTL via both non-cytolytic and cytotoxic mechanisms, is finally responsible for downregulation of hepadnaviral replication and clinical recovery from hepatitis. Furthermore, manipulations of the antiviral immune response in HBV-transgenic mice have suggested an involvement of innate immune cell subsets in inhibition of viral replication (Kakimi et al., 2000; Kakimi et al., 2001). Previous observations in HBV-infected chimpanzees (Wieland et al., 2004b) and WHV-infected woodchucks (Hodgson et al., 1999) have also suggested an involvement of the innate immune system in controlling hepadnavirus infection. However, these studies commenced evaluation of virus replication and intrahepatic immune responses not earlier than one week (Wieland et al., 2004b) post-infection. It is expected, based on the data from experimental infections with other viral pathogens (Biron et al., 1999), that the recognition of the early virological and immunological events could be paramount to a better understanding of the typically observed features of hepadnaviral infection.

Since the half-life of HBV in serum is estimated to be as short as 4 hours (Murray et al., 2006), it could be expected that infection of hepatocytes may occur promptly after exposure to virus, at least in situations when the host is exposed to large, liver pathogenic doses of virus exceeding 10^3 - 10^4 virions (Michalak et al., 2004). Furthermore, it is acknowledged that cells of the innate immune system are activated within minutes to hours following invasion with viral pathogens (Biron et al., 1999). Taken together, we hypothesized that inoculation with WHV at doses known to induce serologically and histologically evident AH (Michalak et al., 2004), should result in

immediate infection of the liver and activation of the hepatic innate immunity, although one previous study on this subject performed in chimpanzees experimentally infected with HBV, in which expression of the innate immune response-affiliated genes was assessed by cDNA microarray analysis, concluded otherwise (Wieland et al., 2004a). To investigate these issues, a large cohort of WHV-naïve woodchucks was infected with a well-characterized WHV inoculum containing 1.1×10^{10} DNase-protected vge per dose. This large cohort of animals permitted reliable determination of viral kinetics and the status of intrahepatic immune activation starting from one hour p.i.

Our quantitative analysis applying a real-time PCR assay demonstrated WHV DNA in serum ($\sim 4 \times 10^6$ vge/ml) and liver tissue ($\sim 6 \times 10^5$ vge/ μ g total DNA) at one hour after injection with virus. However, these high levels of the WHV genomes detected almost certainly originated from the inoculum rather than as a result of an already established active virus replication. On the other hand, quantification of WHV RNA provided a direct insight into the status of virus replication in hepatic tissue. WHV transcripts were found at levels approximating 200 - 400 copies per μ g RNA as early as one hour p.i. This indicated that the virus was in fact able to enter cells within one hour, repair its partially double-stranded DNA, and transcribe DNA to mRNA. In this regard, our study is the first where the evidence of such early replication of hepadnavirus *in vivo* has been documented. In our work, hepadnavirus mRNA was detected by sensitive real-time RT-PCR (sensitivity of <200 vge/ μ g RNA), as opposed to an RNase-protection assay used in one other pertinent study performed in HBV-infected chimpanzees (Wieland et al., 2004a). In this study, chimpanzees showed HBV pregenomic RNA transcripts in the liver beginning from 3 – 4 weeks p.i., with

subsequent exponential expansion between weeks 4 - 6 p.i. The difference between ours and the study mentioned above is almost certainly due to the greater sensitivity of our PCR-based WHV mRNA detection assay.

Our attempt to detect WHV cccDNA in the liver in the first few hours after inoculation was not successful. This replicative intermediate of WHV genome, which constitutes an obligate prerequisite for the generation of hepadnaviral mRNA transcripts (Mason et al., 1983), was identified at 18 hours p.i. The discrepancy between the time of the first detection of WHV cccDNA and WHV mRNA was most likely a consequence of approximately a 10-fold higher sensitivity of the RT-PCR assay used for detection of viral mRNA than that of the PCR assay available for identification of WHV cccDNA ($\sim 1 \times 10^3$ copies/ μg of total DNA).

While the hepatic load of WHV mRNA transcripts progressively increased starting from one hour p.i., there was only a slight parallel increase in the WHV DNA level until 3 weeks p.i., when compared with that detected at 48-72 hours p.i. From week 3 to 6 weeks p.i., a strong coordinated expansion of hepadnaviral genomes and their replicative intermediates was apparent, suggesting exponential viral replication. This result remains in agreement with previous findings from HBV infection showing that exponential viral replication includes proportional increases in expression of both hepadnaviral genomes and replicative intermediates (Murray et al., 2005; Wieland et al., 2004b). Subsequently, a parallel increase in viral RNA transcripts and DNA, although of a lower magnitude, continued until 8-9 weeks p.i. However, there was noticeable transient, but overall statistically insignificant, decrease in the detection of both nucleic acid forms in the liver and WHV DNA in serum around week 6 p.i. in the

majority of the animals. From week 10 p.i. forward, progressive decline in hepatic loads of WHV RNA and DNA occurred lasting until 30 wks p.i. Nonetheless, traces of WHV DNA and RNA remained detectable in hepatic tissue until the end of the observation period, which was as long as 3 years p.i. in some animals. This finding was not surprising and was consistent with the results of our previous studies where the life-long persistence of low-level replication of infectious WHV after seemingly complete serological and biochemical resolution of AH was documented (Hodgson et al., 1999; Hodgson and Michalak, 2001; Michalak et al., 2004; Michalak et al., 1999).

The microenvironment of the liver displays unique immunological properties which have been ascribed to hepatic antigen presenting cells, including liver sinusoidal endothelial cells and Kupffer cells, as well as to the disproportionate occurrence of NK and NKT cells (reviewed in (Racanelli and Rehermann, 2006). Furthermore, recruitment of NK and NKT cells into the liver from the splenic compartment has been observed following viral infection (Salazar-Mather and Hokeness, 2003). Our results suggested that hepadnaviral infection resulted in apparent sequential activation of APC and innate immune effectors within the liver, leading to a decrease in viral burden. Specifically, a significant ($P<0.004$) one-log decrease in hepatic WHV DNA load was detected in all animals which livers were sampled between 48 – 72 hours post infection. The hepatic WHV DNA level returned to that seen prior to this log-decrease 24 hours later, suggesting that the rebound was due to active WHV replication. This temporary reduction of WHV DNA in the liver, and to a lesser degree in serum, was accompanied by a parallel upregulated transcription of cellular markers CD1d and CD3, anti-viral mediators IFN γ , TNF α and OAS, and cytolytic effector molecules

CD95L and perforin. This strongly suggested that a transient activation of intrahepatic innate immunity was able to facilitate a brief but significant decline in the liver virus load, although did not apparently modify hepatic virus transcription. These results are in contrast to those reported by Wieland et al. (2004) which suggested that HBV infection of chimpanzees did not induce activation of intrahepatic innate immunity, as determined by microarray analyses. However, our study commenced evaluation of the host response to hepadnaviral infection within 1 hour of virus inoculation, while the aforementioned study began analysis of liver biopsy tissue obtained beginning at 2 weeks after inoculation with HBV.

Experimental evidence accumulated clearly indicate that NK and NKT cells have the ability to respond to virus by secretion of IFN γ or by acquisition of a cytotoxic function within minutes or hours after infection (Biron et al., 1999). In this regard, recognition of viral antigens by the activating NK receptor NKp46 has been implicated as a key stimulus during influenza virus infection (Mandelboim et al., 2001). Our data demonstrated that as early as 3 to 6 hours after exposure to WHV, there was increased intrahepatic expression of the NK cell marker, NKp46, as well as a significant increase in IFN γ mRNA. Together with an increased perforin expression, a key effector molecule mediating NK cell cytotoxicity, this argues that NK cells were activated almost immediately following hepadnaviral infection. This set of events occurred in parallel with a significant upregulated expression of IL-12, further suggesting that the initial production of IFN γ augmented the activation of APC leading to enhanced expression of a key antigen-presenting molecule CD1d.

Based on detection of WHV transcription shortly after exposure and that hepadnaviral antigens, including large, middle and small envelope proteins, may activate NKT cells (Baron et al., 2002), it is reasonable to suggest that early activation of NK cells could lead to enhanced presentation of WHV antigens to CD1d-restricted NKT cells, culminating in elevations of intrahepatic IFN γ and IL-4 at 48 – 72 hours post-infection. In support of this possibility, CD3 expression was also found to exhibit a distinct peak (a 2.9-fold increase comparing to the pre-infection level) at 48 – 72 hours p.i.) (Fig. 5A, left panel). Since CD1d-restricted NKT cells express a T cell receptor (TCR) comprised of TCR α/β chains in combination with the CD3 receptor complex (Kronenberg and Gapin, 2002), significant elevations in CD3, but not CD4 or CD8 T cell markers, could be interpreted that WHV infection also transiently activated intrahepatic NKT cells leading to the increased expression of IFN γ . Artificial activation of CD1d-restricted NKT cells has been shown to downregulate viral replication in HBV-transgenic mice *via* noncytopathic mechanisms mediated by IFN γ (Kakimi et al., 2000). Overall, ours is the first study demonstrating that hepadnavirus activates innate immune response in the liver shortly after invasion. The results obtained also suggest that hepadnaviral infection may first activate NK cells and subsequently NKT cells, with the latter possibly contributing to a transient decrease in viral DNA. The activation of intrahepatic innate immunity is transient, waning by 72 hours post infection, and was therefore undetected in previous studies which commenced evaluation of anti-viral immunity at one (Wieland et al., 2004b) or 2 weeks (Wieland et al., 2004a) post inoculation.

LCMV infection has been shown to induce NKT cell activation, resulting in IFN γ and IL-4 expression, which is immediately followed by a decrease in their levels and subsequent increase several weeks later (Hobbs et al., 2001; Lin et al., 2005b). Interestingly, peak expression of the NK marker, NKp46, occurred in our study at 3 weeks p.i., coinciding with significant elevations in IL-4 expression, in the absence of upregulated expression of IFN γ . Although we cannot determine the cellular site of IL-4 augmented expression, the histological analysis showed a lack of lymphomononuclear infiltrations at this time point in the liver. Thus, NKT cells may represent the predominant cell type responsible for the increased hepatic expression of IL-4 in our experiment. Since woodchuck-specific antibodies for detection of NKT cells or IL-4 are currently lacking, the explanation of this possibility will require further investigation when such reagents become available. Furthermore, WHV-mediated skewing of the immune response towards a T_H2-type, characterized by IL-4 and the absence of IFN γ , is another enticing possibility. Our data could suggest that transient activation of T_H2-type responses, coinciding with detectable levels of WHsAg in circulation at 3 weeks p.i., may enable uninhibited viral expansion prior to activation of T_H1-type adaptive immunity and infiltration of virus-specific T cells into the infected liver.

Despite evident activation of intrahepatic innate immune cell subsets during the first hours and days following infection, coinciding with significant reductions in viral load, this initial anti-viral response waned and failed to promptly induce T cell response, characterized by CD4 and CD8 T cell infiltrations into the liver, until 5 to 6 weeks later. These findings are in direct contrast to those observed during infections

with other viral pathogens which induce timely sequential activation of innate and adaptive immune cell subsets leading in many cases to the self-resolution of the infection. The prolonged period of immunological ignorance to hepadnaviral infection, as characterized in our study by a lack of expression of T cell marker genes and liver inflammatory infiltrations following activation of the intrahepatic innate immune response or by others as a lack of T cell specific responses to viral antigens (Bertoletti and Ferrari, 2003; Menne et al., 1998), may be partially explained by the tolerizing effect of the liver. The capacity of the liver to induce tolerance to oral or allogenic antigens is now well recognized (Berg et al., 2006; Limmer et al., 2005). It is understood that it occurs via several mechanisms, including suboptimal T cell priming and induction of T cell anergy (reviewed in (Bertolino et al., 2002; Crispe, 2003). In our study, the expression of CD4 and CD8 T cell co-receptors was transiently elevated immediately following infection. In addition, inflammatory mediators, including IFN γ , have been shown to influence the expression of adhesion molecules on endothelial cells, which have been implicated in mediating T cell trapping in the liver (Mehal et al., 2001). Furthermore, transient activation of antigen-specific T cells has been observed in TCR-transgenic mouse models, wherein cognate antigen presentation was restricted to hepatocytes, leading to dysfunctional priming of naïve T cells (Bertolino et al., 2001; Bertolino et al., 1998). Thus, initial trapping of CD4 and CD8 T cells, followed by suboptimal priming or deletion, may potentially facilitate hepadnaviral subversion of the adaptive immune response.

Overall, our data show that hepadnaviral replication is not quiescent during the phase immediately following virus invasion but progresses at low level in the liver

beginning from one hour after exposure to virus. Furthermore, hepadnaviral infection stimulates almost immediately intrahepatic innate immune cells, including APC, NK and NKT cells, which cause a transient downregulation of virus hepatic load. These findings are in contrast with those recently reported for experimental HBV infection in chimpanzees, which suggested a lack of innate immune activation after exposure to infectious hepadnavirus as reported by Wieland *et al.* (2004). However, there are several important differences between our study and that in chimpanzees. First, the study mentioned, despite the intention to analyze whether HBV infection activates innate immunity in acutely-infected chimpanzees, commenced analysis of intrahepatic immune activation at two weeks p.i. As the data obtained in our study showed, the activation of this immunity has occurred and waned by one week p.i. Secondly, analysis of a small number of HBV-infected chimpanzees was done by cDNA microarrays (Wieland et al., 2004a). Thus, examination of relatively a small group of animals by a technique of a lower sensitivity than real-time RT-PCR (approximately by 5-10-fold) may have additionally contributed to omission of biologically important mediators or cellular markers. This was evidenced and realized in the work mentioned where a combination of the data from microarray analysis and the definitions used to include or exclude particular genes failed to include IFN γ as a mediator of hepadnaviral clearance (Wieland et al., 2004a).

In summary, our data indicate that hepadnaviral infection induced by a liver pathogenic virus dose is characterized by a rapid initiation of viral replication in the liver and activation of the local innate immune system. This early intrahepatic immune reactivity is capable of a profound downregulation of hepatic virus load, however, is

transient and, in contrast with other viral infections, does not precipitate a swift adaptive immune response. A reason behind this inability is unknown and could be due to as yet unidentified viral factors or as a possible consequence of the inherent tolerizing action of the liver.

CHAPTER SIX: GENERAL DISCUSSION AND FUTURE DIRECTIONS

The main purpose of the present series of studies was to investigate how the intrahepatic environment may influence the immunological response to hepadnaviral infection, with the following two considerations: (1) the liver is inherently immune tolerizing by as yet incompletely understood mechanisms and (2) the intrahepatic microenvironment is uniquely enriched for cells of the innate immune system which may rapidly recognize and respond to pathogens.

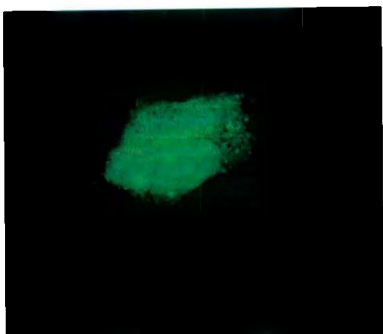
The contribution of the liver towards maintenance of peripheral tolerance against oral or transplanted antigens is increasingly recognized (reviewed in Crispe, 2003). Furthermore, studies suggest that the liver represents a final repository for activated lymphocytes (Huang et al., 1994). While Kupffer cells and sinusoidal endothelial cells have been found to contribute to tolerogenic mechanisms and removal of activated cells, we investigated in our initial study whether hepatocytes may potentially contribute to cell removal via the intrinsic expression of CD95L. We demonstrated that cultured, as well as freshly isolated, primary hepatocytes derived from healthy human and woodchuck livers inherently express biologically active CD95L capable of inducing killing of CD95-positive cells. Interestingly, stimulation by exogenous IFN γ or TNF α resulted in an upregulated expression of CD95L by hepatocytes. These results challenged the previous view that hepatocytes were simply passive targets of activated cytotoxic lymphocytes, and suggested that they may work co-operatively with Kupffer or LSECs to mediate trapping and removal of activated lymphocytes as well as other cells. Increased hepatocyte expression of CD95L in response to inflammatory cytokines may suggest a contribution of hepatocytes towards

the development of liver disease, including viral hepatitis characterized by inflammatory lymphomononuclear infiltrates which secrete IFN γ and TNF α .

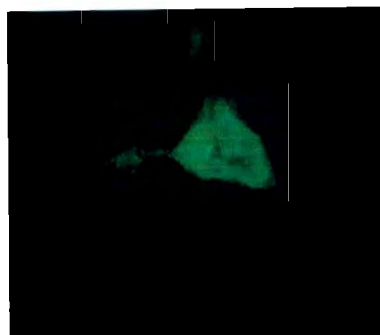
Hepatocytes are sensitive to CD95-mediated apoptosis (Ogasawara et al., 1993). The failure of hepatocyte-expressed CD95L to initiate autocidal or fratricidal death under normal physiological conditions may be reconciled by the segregated expression of ligand and receptor. It has been demonstrated in lymphoid cells that CD95L is contained within secretory granules and can be localized to the plasma membrane upon target cell recognition and degranulation (Kojima et al., 2002). In contrast, epithelial-derived cell lines have been shown to transport translated CD95L directly to the plasma membrane (Blott et al., 2001). To determine how CD95L is sorted in hepatocytes, we constructed a plasmid containing full length mouse CD95L, fused at its amino terminus to the reporter green fluorescent protein (GFP). Since CD95L is a type II transmembrane protein, fusion of GFP to the amino terminus would result in cytoplasmic retention of the reporter protein upon membrane expression and cleavage of CD95L by extracellular metalloproteinases (Kayagaki et al., 1995). In contrast with 293HEK cells, which rapidly transport GFP-fused CD95L to the plasma membrane, leading to apoptosis of transfected cells (data not shown), WCM-260 hepatocytes retain the GFP-CD95L fusion protein within the cytoplasm and their survival was not adversely affected (Figure 6.1). Thus, while WCM-260 hepatocytes are susceptible to apoptosis mediated by anti-CD95 antibody treatment (data not shown), they are resistant to CD95L-mediated lysis when the ligand is expressed by hepatocytes themselves. Whether inflammatory cytokines modulate the intra- or extracellular expression of CD95L by hepatocytes requires further investigation.

Fig 6.1. GFP-CD95L fusion protein exhibits cytoplasmic localization in WCM-260 hepatocytes. Full length cDNA encoding murine CD95L was inserted into the pEGFP-C2 eukaryotic expression plasmid designed to generate a GFP-CD95L fusion protein in transfected cells. WCM-260 hepatocytes were cultured to >90 % confluence on glass coverslips and were transiently transfected with empty pEGFP-C2 vector (A and B), or with pEGFP-C2-CD95L vector encoding the GFP-CD95L fusion protein (C and D). Following 24 hours culture, cells were fixed in 2 % paraformaldehyde and imaged using laser scanning confocal microscopy. Arrows indicate intracytoplasmic GFP-positive structures suggesting CD95L localization in cytoplasmic vesicles.

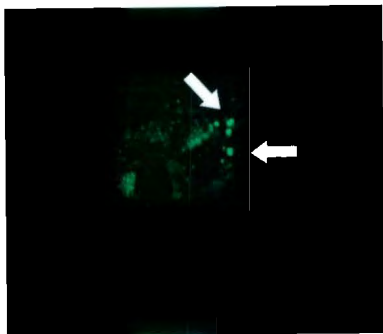
A



B



C



D

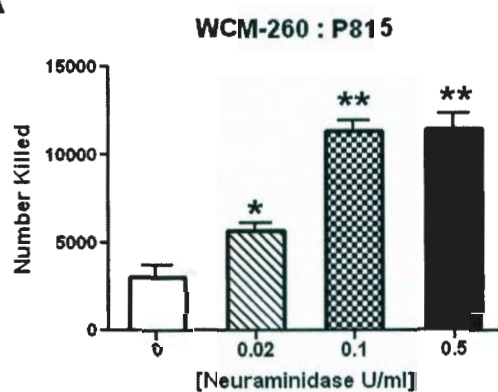
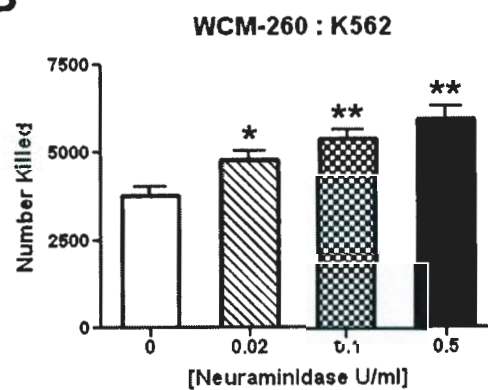
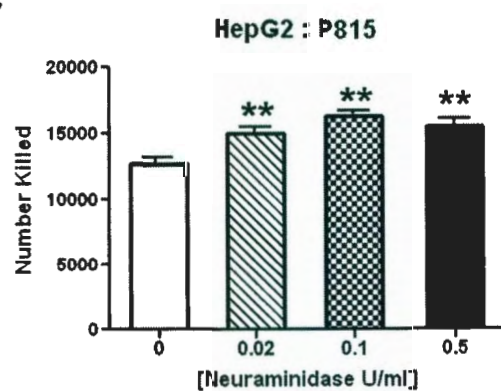
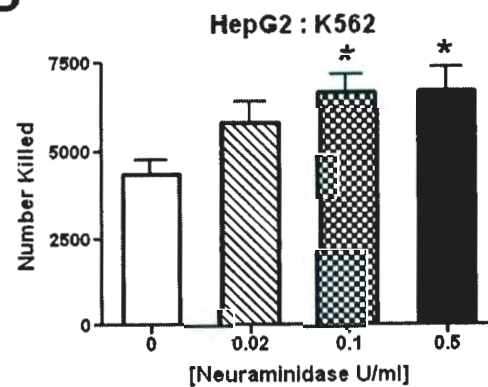


Although our initial study demonstrated that hepatocyte expression of CD95L was at least in part responsible for inducing apoptosis of CD95-sensitive targets, inhibition experiments suggested that an additional cytotoxic pathway was concomitantly utilized by hepatocytes. The expression of components comprising the perforin/granzyme B cytolytic pathway has been previously determined to be restricted to cells of the immune system, primarily CD8⁺ CTL and NK or NKT cells. Our second study hypothesized that hepatocytes may also utilize the perforin pathway, alone or in combination with CD95L, to induce death of contacted cells. In this study, we found that hepatocytes express mRNA encoding perforin and granzyme B. Perforin protein was detectable in lysates derived from hepatocyte cell lines and freshly isolated primary hepatocytes by Western blotting, while flow cytometric analysis confirmed that perforin expression was readily identifiable in these cells. The perforin cytotoxic pathway was determined to be biologically active, since hepatocytes mediated death of CD95-negative cells in a manner which could be at least partially attenuated by perforin-specific neutralizing antibodies or shRNA. In contrast with CD95L, IFN γ or TNF α were unable to influence hepatocyte expression of perforin or cytotoxicity mediated by the perforin pathway. Thus, we concluded that normal hepatocytes are constitutively able to remove other cells via either the CD95L- and/or perforin- pathways, while inflammatory cytokines may differentially modulate the expression of CD95L but not perforin by hepatocyte effectors.

Although we have demonstrated that hepatocytes express biologically active CD95L and perforin effector molecules, it remains undetermined how hepatocytes recognize targeted cells, or whether hepatocytes exhibit indiscriminate cytotoxic

activity. CTL mediate directed lysis of target cells following recognition and binding of antigen, in the context of MHC class I molecules, to the TCR. NK cells exhibit antigen-independent lysis of cell targets, however, the discrimination between target and non-target cells is facilitated by the balance of activating or inhibiting ligands expressed by the target which trigger or inhibit, respectively, activation of the NK cell. We hypothesize that hepatocytes are not indiscriminate cytotoxic effectors, but that they recognize target cells via an antigen-independent yet selective mechanism, in part analogous to NK cell effectors. ASGPR is essentially a hepatocyte-specific receptor which recognizes terminally desialylated glycoproteins. It has been shown that treatment of lymphocytes with neuraminidase, an enzyme which removes terminal sialic acid residues from glycoproteins, results in retention and removal of lymphocytes by the liver (Samlowski et al., 1984). The mechanism leading to lymphocyte removal was previously undetermined, but it was hypothesized that the *in vitro*-activated and subsequently transferred T cells underwent autocidal or fratricidal lysis. Using cultured WCM-260 hepatocytes or HepG2 cells, we have found that neuraminidase treatment of CD95-bearing P815 (Figure 6.2A and C) or CD95-deficient K562 targets (Figure 6.2B and D) leads to a dose-dependent increase in the level of target cell lysis. We propose that ASGPR-mediated recognition of target cells facilitates discrimination and removal of activated lymphocytes expressing the activated form of CD45 (i.e., the B220 epitope) by hepatocytes.

Since we had found that hepatocytes were inherently capable of removing CD95-bearing or CD95-deficient cells, we further investigated whether hepadnaviral infection and accompanying liver inflammation could modulate the cytotoxic potential

A**B****C****D**

of hepatocytes. Results from our initial studies would predict that inflammation associated with chronic viral hepatitis, or that continuing minimal to moderate inflammation observed after recovery from AH, would modulate hepatocyte expression of CD95L, but not perforin. As expected, and in agreement with previous studies from this laboratory (Hodgson and Michalak, 2001), a slight but significant and continued elevation in intrahepatic expression of IFN γ followed recovery from acute WHV hepatitis. This was associated with increased hepatocyte expression of CD95L. Despite a greater degree of inflammation and increased intrahepatic levels of IFN γ and TNF α during chronic WHV hepatitis, hepatocyte expression of CD95L was not increased relative to that observed after recovery from acute infection. This suggests that a relatively moderate increase in liver inflammation may yield maximal upregulation of CD95L expression by hepatocytes.

Surprisingly, hepadnaviral infection also invariably increased hepatocyte expression of perforin, and perforin-dependent capacity to kill CD95-deficient cells. Since in vitro data suggested that perforin expression in hepatocytes and lymphoid cells was differentially influenced, with hepatocytes displaying resistance to IFN γ -mediated increases, we hypothesize that as yet unknown mediators are responsible for upregulating perforin expression upon hepadnaviral challenge.

The functional consequences of increased hepatocyte cytotoxic potential, as a result of resolved or chronic hepadnaviral hepatitis, are as yet unrecognized. Virus-specific T cell responses are detectable during extended follow-up of patients which have resolved acute HBV infection (Rehermann et al., 1996). Elevations of intrahepatic IFN γ and CD3 mRNAs are consistently detectable for years following

recovery from acute WHV hepatitis (Hodgson and Michalak, 2001), and they are likely reflective of continued immune activation due to low-level viral replication and production of viral antigens (Michalak et al., 1999). It is possible that increased hepatocyte cytotoxicity during recovery from hepadnaviral infection, may represent a physiological mechanism to protect hepatocytes from more extensive injury due to minimal to moderate continuous lymphomononuclear cell infiltrations by removing activated T cells.

It is understood that the development of chronic hepadnaviral hepatitis is at least in part due to an insufficient anti-viral T cell response which is unable to inhibit viral replication through cytolytic and non-cytolytic mechanisms. In contrast with SLAH, CH is characterized by the persistence of evident inflammatory infiltrates and hepatocellular injury. In such a scenario, the hypothesized protection afforded the hepatocytes by cytokine-mediated upregulation of cytotoxic mechanisms, may be insufficient to prevent hepatocyte injury occurring as a result of overwhelming bystander recruitment of non-specific, activated lymphocytes and other immune cells.

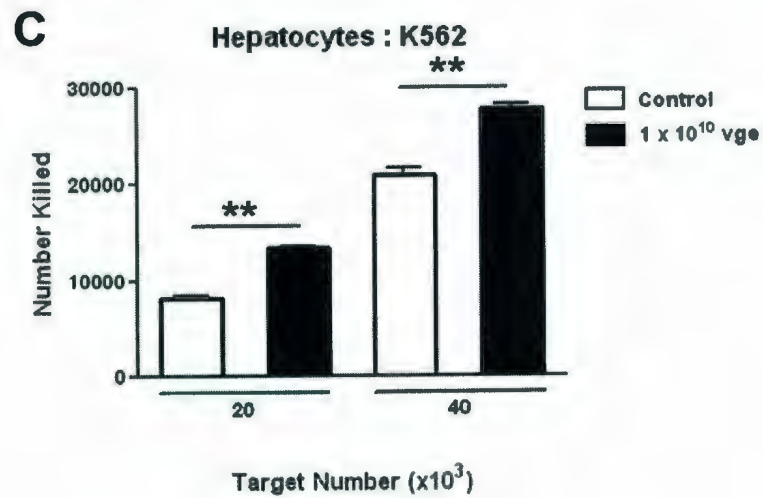
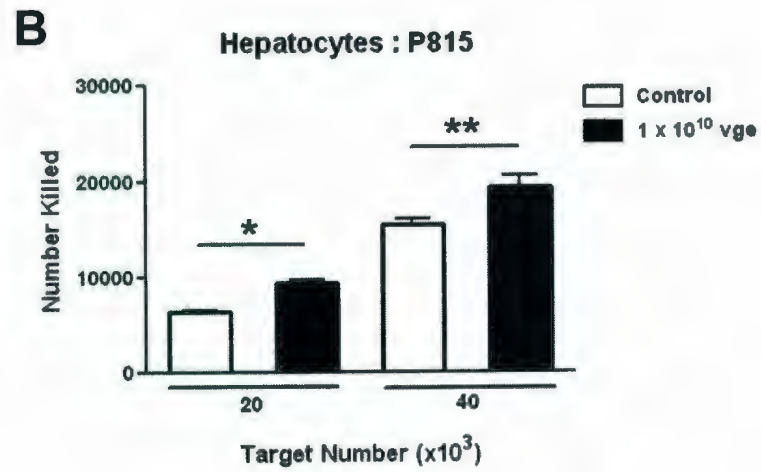
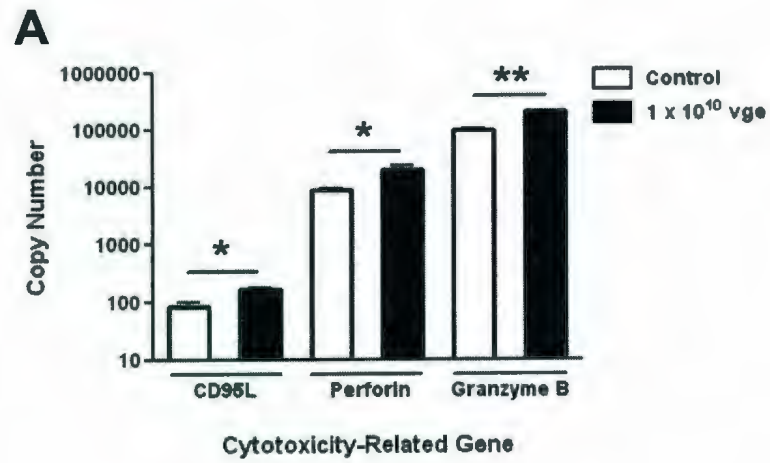
On the other hand, functional expression of CD95L and perforin effector molecules by hepatocytes may also contribute to liver pathology. The development of several liver diseases, including alcoholic hepatitis (Galle et al., 1995), Wilson's disease (Strand et al., 1998) and HCC (Roskams et al., 2000) is associated with increased hepatocyte expression of CD95L. While constitutive expression of CD95L or perforin may serve a physiological role for maintaining tolerance or removal of activated T cells, as discussed previously, increase in these cytotoxic activities may contribute to liver injury.

Having demonstrated that hepadnaviral infection and accompanied inflammation were associated with increased cytolytic capability of hepatocytes, thereby possibly contributing to shaping of the immune response to hepadnavirus via tolerogenic or pathologic mechanisms, we further evaluated if the intrahepatic innate immune system is activated by hepadnavirus infection and may contribute to local antiviral immunity. The liver is characterized immunologically by its unique composition of innate immune effector cells (NK and NKT), unconventional T cell subsets, and an endothelium which displays inherent capacity to scavenge and present antigens to naïve immune effectors. Initial studies in HBV-transgenic mice (Kakimi et al., 2000), suggested an involvement of NKT cells in the downregulation of HBV replication, however, confirmation that innate immune effectors are activated during hepadnaviral infection had not been extensively or appropriately undertaken in a natural model. Specifically, IFN γ -mediated downregulation of HBV-infected chimpanzees appeared to precede T cell infiltration into the liver (Guidotti et al., 1999) however the immune cell subsets responsible for expression of IFN γ remained undetermined. Subsequent microarray analysis of intrahepatic gene expression in HBV-infected chimpanzees commenced at two weeks p.i. (Wieland et al., 2004a). It was concluded that HBV-infection does not activate intrahepatic innate immunity, however it remained conceivable that anti-viral innate immunity may have been initiated, and waned, prior to the initial sample collection at two weeks p.i. Using a large cohort of WHV-infected woodchucks, we were able to analyze viral kinetics and the intrahepatic immune response beginning at one hour post infection. The data clearly indicated that WHV is capable of entry and initiation of transcription as early as

1 hour after inoculation. WHV mRNA exhibited a progressive increase in the liver beginning from one hour post-exposure to virus. This may suggest that produced viral antigens or RNA species may in turn activate intrahepatic APC resulting in subsequent elevations of the T_H1 -polarizing cytokine IL-12, as early as 3 – 6 hours p.i. Not surprisingly, given the known reciprocal activation of NK cells and APC, elevations in IFN γ , perforin, NKp46 and IL-4 expression were found, suggesting activation of NK and NKT cells at 48 – 72 hours post WHV infection. In contrast with infection with other viral pathogens, activation of intrahepatic innate immune cells did not lead to significant activation of conventional CD4⁺ or CD8⁺ T cells at early time points post-inoculation, and their involvement did not become evident until the peak of WHV-induced liver disease 5 – 6 weeks post-viral exposure.

Interestingly, the obtained data suggested that cytotoxic mechanisms were possibly upregulated at these early time points in the liver since both CD95L and perforin mRNA expression were elevated. While perforin expression may be upregulated in activated NK or NKT cells, it remained undetermined, based on RT-PCR analysis of cDNA derived from liver biopsy tissue, if increased CD95L or perforin mRNAs were reflective of increased hepatocyte cytotoxic potential. To investigate this latter possibility, a second group of healthy woodchucks was challenged with a large infectious dose of WHV and were subsequently euthanized at 72 hours p.i., facilitating isolation of hepatocytes and analysis of their cytotoxic potential. Hepadnaviral infection resulted in an upregulation of CD95L as well as perforin and granzyme B mRNAs (Figure 6.3A), and an increased ability of

Fig 6.3. Hepadnavirus augments hepatocyte-mediated cytotoxicity 72 hours p.i. Primary hepatocytes were isolated from adult woodchucks ($n = 2$) at 72 hours p.i. with 1×10^{10} WHV vge, or from healthy control animals ($n = 2$). Gene expression levels were quantified by real-time RT-PCR and were normalized against expression of β -actin (A). The cytotoxic capacity of hepatocytes (6×10^4 /well) was examined by JAM DNA fragmentation assays using 4×10^4 CD95-bearing P815 cells (B) and CD95-deficient K562 cell targets (C). Results are shown as mean values \pm SEM of two animals per group, with each experiment performed with 8 - 16 experimental wells per condition. Data bars marked with * are significant at $P < 0.005$ and those with ** at $P < 0.0001$ when compared with healthy controls.



hepatocytes to lyse both CD95-bearing (Figure 6.3B) and CD95-deficient targets (Figure 6.3C), when compared with healthy controls.

This series of studies suggest that hepatocyte cytotoxicity may in fact contribute to hepadnaviral infection by removal of activated, virus-specific T cells in the liver.

Hepatocyte-mediated removal of activated T cells may represent a component of the tolerogenic capability of the liver. Previous studies in bone marrow chimeric mice which express a MHC class I-restricted antigen on hepatocytes but not on APC, indicated that naïve, antigen-specific CD8⁺ T cells accumulate in the liver where they exhibit transient activation followed by premature death by apoptosis (Bertolino et al., 1998). Subsequent investigations in this model revealed that this effect was specific for CD8⁺ T cells, did not require help from CD4⁺ T cells or APC, and was dependent on the expression of adhesion molecules by the liver endothelium or hepatocytes (Bertolino et al., 2005; Bertolino et al., 1998). It was confirmed that the trapped CTL were antigen-specific (Bertolino et al., 2005), and were activated, as determined by their expression of B220 or the desialylated isoform of CD45 which is capable of binding to hepatocyte ASGPR. Considering the results of these studies and our in vitro data, which demonstrate that target cells expressing desialylated glycoproteins are more efficiently targeted by hepatocytes, we propose that hepatocytes are able to transiently activate naïve T cells, but in contrast with their activation by professional APC, their activation is dysfunctional and is promptly followed by hepatocyte-mediated T-cell apoptosis. A recent study utilizing transgenic mice which display a cognate CTL antigen ubiquitously (Met-K^b) or restricted to hepatocytes (Alb-K^b), suggests that primary activation of antigen-specific naïve T cells by professional APC leads to

hepatitis, while primary activation of the same cells by hepatocytes leads to tolerance (Bowen et al., 2004). Failure of adoptively transferred naïve CTL to mediate hepatitis in Alb-K^b mice was due to reduced CTL survival, despite similar rates of initial activation as those observed in Met-K^b mice, possibly due to active removal mediated by hepatocytes. These findings may be of significance to the development of intrahepatic immune responses following hepadnaviral infection or infections with other hepatotropic pathogens.

Our data clearly indicate that exposure to liver pathogenic WHV is followed by immediate activation of viral transcription and strongly suggests sequential activation of APC and NK or NKT cell subsets. Despite apparent activation of innate immune cell subsets, which by convention precedes activation of adaptive T cell responses, significant intrahepatic infiltrations of T cells was not observed until several weeks following infection. We would propose that initial intrahepatic recognition of viral antigens by naïve T cells which contact virus-infected hepatocytes results in transient activation and removal, despite an appropriate microenvironment characterized by the presence of activated APC and innate immune subsets. This potential tolerizing effect of the liver is overcome several weeks later (Menne et al., 1998), as evidenced by peripheral T cell responses directed against WHsAg and WHcAg. Our data suggest that exponential virus replication and translation of virus proteins beginning at 3 weeks p.i., may facilitate extrahepatic lymph-node or splenic priming of T cells which, in contrast with T cells which are initially activated by hepatocytes (Bowen et al., 2004), are functionally capable of infiltrating the liver and downregulating virus replication while mediating clinically evident hepatitis.

Taken together and in the context of current literature, our studies suggest that constitutive expression of CD95L and perforin effector molecules by hepatocytes may contribute to the inherent tolerizing capability of the liver. Hepatic tolerance likely includes contributions from resident Kupffer cells (Mehal et al., 2001), LSEC (Knolle and Limmer, 2001), and hepatocytes which facilitate trapping and removal of systemically-activated lymphocytes which enter the liver. Furthermore, the ability of hepatocytes to transiently activate naïve T cells and induce cell death via apoptosis, may represent a physiological mechanism which also maintains peripheral tolerance to dietary or transplanted antigens. However, hepatotropic pathogens, many of which establish chronic infections, including hepadnaviruses, may usurp this protective mechanism, leading to removal of pathogen-specific effector T cells following antigen-specific trapping (Bertolino et al., 2001; Bertolino et al., 2005; Bowen et al., 2004) and facilitating the expansion of virus replication. Hepadnaviral infection as well as other inflammatory liver diseases may influence hepatocyte expression of CD95L and perforin effector molecules with as yet unrecognized consequences. Although increased hepatocyte cytotoxicity may facilitate removal of activated T cells and facilitate to some extent protection of the liver from pathogenic T cell infiltrations, the relative sensitivity of hepatocytes to suicidal CD95L-mediated lysis during liver inflammation is unknown. Furthermore, while hepatocytes appear to sort CD95L to an intracellular compartment under normal culture conditions, it is possible that hepadnavirus infection may modulate the cellular expression of the ligand or CD95 receptor. For example, upregulation of CD95L by the WHV X gene may suggest that integration of this viral gene sequence into the genome may contribute to the

development of HCC through cytolytic mechanisms mediated by CD95L-CD95 interactions. These possibilities require further investigation to fully understand the importance of the cytotoxic potential of hepatocytes during the initial intrahepatic response to hepadnavirus, recovery from hepadnaviral infection, or the development of chronic viral hepatitis.

6.1 FUTURE DIRECTIONS

The results of the current studies have further supported the potential importance of the intrahepatic microenvironment on regulation of peripheral immune responses in general, and in hepadnaviral infection, in particular. These findings raise the following issues and, among others, impose several intriguing questions:

1. We have demonstrated that hepatocytes are constitutively capable of inducing death of target cells via CD95L or perforin pathways. CD95L and perforin have been determined to co-localize in secretory granules in CTL. Although preliminary data suggest that CD95L is sorted to an intracellular compartment in cultured hepatocytes, it remains unresolved if perforin and associated proteases, such as granzyme B, are coordinately expressed in the same cellular location and if this phenomenon holds true for primary hepatocytes. While we have found that neuraminidase-treated targets are more susceptible to hepatocyte-mediated lysis, suggesting an involvement of ASGPR-mediated recognition of targeted cells, key questions to be investigated are whether hepatocytes express multiple receptors capable of target cell recognition, and how do

these receptors harness components of the cellular cytoskeleton to mediate release of intracellular effector molecules?

2. IFN γ , and to a lesser extent TNF α , were found to increase CD95L expression and upregulate hepatocyte-mediated CD95L cytotoxicity. It remains unrecognized, however, if increased lysis of CD95-sensitive target cells following stimulation of hepatocytes with inflammatory cytokines is due simply to greater expression of the ligand, or whether IFN γ or TNF α may concomitantly increase the capability of hepatocytes to recognize target cells. Following our initial discovery that ASGPR may facilitate recognition of target cells, examination of cytokine-mediated regulation of ASGPR expression is warranted. Furthermore, investigations using shRNA directed against either ASGPR subunit would permit testing of the hypothesis that ASGPR is responsible for target cell recognition, in the presence or absence of inflammatory conditions.

3. In contrast with cytokine-mediated upregulation of CD95L, exposure of hepatocytes to IFN γ or TNF α did not influence perforin expression. However, hepadnaviral infection invariably increased the capability of hepatocytes to lyse both CD95-sensitive and unexpectedly, CD95-insensitive cells. Additional studies are required to determine if: (1) in addition to increased CD95L expression, do other liver diseases result in increased hepatocyte expression of perforin and (2) what biological mediators are responsible for regulation of perforin expression and utilization by hepatocytes?

4. The capacity of hepatocytes to transiently activate naïve T cells, resulting in their dysfunction and premature death, has been conclusively established (Bertolino et al., 2001; Bowen et al., 2004). Hepatocyte-mediated activation and deletion of HIV-specific T cells may partially explain the observation that virus-specific T cells are undetectable for several weeks p.i. This concept is not readily tested using a natural model of hepadnaviral infection, however, the use of HBV-transgenic mice and TCR-transgenic T cells specific for viral antigens could be used to determine the validity of this hypothesis. Furthermore, generation of HBV-transgenic mice which are also CD95L^{-/-} or perforin^{-/-} would facilitate examination of whether hepatocyte-mediated cytotoxicity is required for induction of T cell apoptosis following transient activation.

5. Our data clearly indicated that activation of intrahepatic innate immune subsets lead to a significant ~10-fold decrease in viral replication very early (48 – 72 hours) post-infection. These are the first results to suggest that NK and NKT cell activation in fact occurs in natural hepadnaviral infection, which is in agreement with the previous observations that artificial activation of NKT cells can downregulate virus replication in HBV-transgenic mice (Kakimi et al., 2000). Identification of the ligand/s responsible for activation of these cells during hepadnaviral infection, may represent an attractive therapeutic approach for potential suppression of hepadnaviral replication in chronic hepatitis.

CHAPTER SEVEN: SUMMARY AND CONCLUSIONS

Over the course of this series of studies, we investigated how hepatocytes may contribute to liver immunological responses and characterized step-by-step the intrahepatic milieu accompanying establishment of hepadnaviral infection and development of hepatitis in search for answers as to how the unique immunological environment of this organ may influence anti-viral responses against hepadnavirus, particularly in the early stages which appear to be critical to the outcome of hepatitis. To study these issues, we applied the woodchuck-WHV infection system, which is the closest animal model of HBV infection and hepatitis B. The results obtained can be summarized and concluded as follows:

1. Molecular and immunoprobng analyses of primary, freshly isolated, normal woodchuck and human hepatocytes, as well as cultured woodchuck hepatocytes and human hepatoma HepG2 cells demonstrated constitutive transcription of CD95L and expression of CD95L protein. Subsequently, we adapted the JAM DNA fragmentation assay, which is conventionally used to assess lymphoid cell-mediated cytotoxicity, to examine the cell killing capability of freshly isolated or cultured hepatocytes. The study demonstrated that normal hepatocytes are constitutively capable of inducing death of CD95-expressing target cells. Utilization of a CD95-specific blocking antibody to prevent CD95L-CD95 interactions, significantly reduced target cell killing and confirmed that hepatocytes utilized CD95L to mediate killing of CD95-bearing cells. Furthermore, it was found that hepatocyte stimulation with either exogenous

IFN γ or TNF α resulted in upregulated expression of CD95L and increased the cytotoxic capability of hepatocytes. These results provided the first ever evidence that hepatocytes may act as cytotoxic effector cells and that the local intrahepatic cytokine environment may augment hepatocyte cell killing potential.

2. During the study on hepatocyte-mediated, CD95L-dependent cytotoxicity, it became apparent that the CD95L-CD95 interaction was not entirely, although to a large degree, responsible for the observed hepatocyte-mediated cell death. Therefore, we investigated whether hepatocytes may utilize the perforin cytotoxic pathway. RT-PCR, Western blot and flow cytometric analyses demonstrated that hepatocytes indeed express perforin mRNA and display perforin protein. In addition, disruption of microtubule polymerization during JAM DNA fragmentation assays suggested that hepatocytes utilize the exocytic pathway to mediate killing of both CD95-bearing and CD95-deficient cell targets. Additionally, neutralizing anti-perforin antibodies and treatment of hepatocytes with perforin-specific shRNA demonstrated that cultured as well as freshly isolated primary hepatocytes utilize perforin to kill target cells. In contrast with CD95L, IFN γ - or TNF α -treatment of hepatocytes failed to modulate perforin expression and increase cell killing. However, IFN γ augmented perforin expression in lymphoid cells, suggesting that regulation of the perforin pathway by this cytokine may be cell-type specific. This study is the first which demonstrates that non-lymphoid cells express perforin and can utilize the exocytic pathway to mediate killing of contacted cell targets. Furthermore, inflammatory cytokines may differentially

influence the usage of CD95L- or perforin-dependent killing mechanisms by hepatocytes.

3. Since we documented that hepatocytes constitutively express biologically active CD95L and perforin effector molecules and may concomitantly utilize both cytolytic pathways to induce target cell death, we investigated whether hepadnaviral infection could alter the cytotoxic phenotype and potency of hepatocyte effectors. We discovered that hepatocytes in animals convalescent from acute WHV infection and in those with progressing chronic WHV hepatitis, transcribed significantly greater quantities of CD95L and perforin in comparison with healthy hepatocytes used as controls. In addition, hepatocytes derived from convalescent or chronically infected animals were able to induce significantly greater killing of both CD95-bearing and CD95-deficient cells. In support of our *in vitro* data, increased liver expression of IFN γ was correlated with increased hepatocyte-mediated, CD95L-dependent cytotoxicity. The results further suggested that transient, minimal to moderate hepatic inflammation, histologically evident during convalescence, was sufficient to maximally increase hepatocyte-mediated cell killing. Also, both resolution of hepadnaviral infection and chronic WHV-induced hepatitis were accompanied by upregulated hepatocyte expression and usage of the perforin cytotoxic pathway. Since *in vitro* data suggested that IFN γ and TNF α do not influence hepatocyte expression of perforin, the mechanisms underlying augmented perforin expression *in vivo* are currently unknown. Moreover, using cultured woodchuck hepatocytes stably expressing complete WHV genomes or individual viral proteins, we have excluded a direct role for hepadnaviral

infection on the modulation of hepatocyte cytotoxicity. Based on the data collected, we concluded that hepatic inflammation induced by hepadnavirus rather than replication of hepadnavirus itself influences the cytotoxic phenotype of hepatocytes.

4. Finally, we analyzed viral replication kinetics and activation of intrahepatic innate and adaptive immune responses following experimental infection with hepadnavirus. This study documented that while WHV RNA transcripts are detectable as early as one hour p.i., exponential replication of the virus does not occur in the liver until several weeks later. Nonetheless, WHV invasion due to inoculation with a large, liver pathogenic virus dose induced the sequential activation of intrahepatic APCs and then NK and NKT cells beginning 3 to 6 hours p.i., which peaked at 48 – 72 hours after exposure to virus and was coincident with a significant decrease in the liver load of WHV genomes. Despite activation of innate immune cell subsets, characterized by increased intrahepatic expression of IL-12 and IFN γ , increased lymphomononuclear infiltrations into the liver were not observed for several weeks and occurred after the exponential expansion of virus replication at 3 – 4 weeks following infection. Isolation of hepatocytes 72 hours p.i. revealed that, in comparison with healthy controls, hepatocytes derived from WHV-infected livers demonstrated significantly greater expression of CD95L and perforin, and possessed increased cytotoxic activity against both CD95-bearing and CD95-deficient target cells. The results of these multiparametric investigations indicate that hepadnavirus replication is promptly established in the liver after exposure to a liver pathogenic dose and that, in contrast to the previous data from HBV-infected chimpanzees, is capable of immediate activation

of hepatic innate immunity. However, this very early innate immune response appears to be insufficient to prime strong adaptive T cell immunity capable of early virus elimination. This might be due to hepatocyte-mediated removal of activated virus-specific T cells or due to yet unrecognized tolerizing properties of the liver in regard to hepadnavirus which naturally replicates within hepatocytes.

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Appendix A

The following appendices include woodchuck cellular gene sequences submitted to GenBank throughout the course of the thesis work.

A.1

EF621770 600 bp mRNA linear ROD 19-AUG-2007
DEFINITION Marmota monax CD40 ligand mRNA, partial cds.
ACCESSION EF621770
SOURCE Marmota monax (woodchuck)
AUTHORS Guy, C.S. and Michalak, T.I.
TITLE Direct Submission
FEATURES Location/Qualifiers
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 /db_xref="taxon:9995"
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ORIGIN

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121 gacaagatag aagatgaaag gaatcttcat gaggatttg tattcatgaa aacgatacag
181 agatgcaaca attgcgatgg atctttatcc ttgctgaact gtgaggaaat ccgaagtcaa
241 ttggaaggct ttgtcaagga tataatgta aacaaaggagg aaaagaagaa agaaagtgtt
301 gaaatgcaaa aaggatgatca gaatccacaa attgctgtac atgttgtaag tgaggccagt
361 aaaacagcat ctgtgtctaca gtgggccaaa aaaggatatt acaccatgag caacaacttg
421 gtaacccttg aaaatgggaa acagctgact gtaaaagag aaggactcta ttatgtctac
481 acccaagtta cttctgttc caaccgggaa gctttgagtc aagctccatt tatagtcagc
541 ctctgtctga agtcctcaag tgaatccgag agaactctac tgaggcgggc aaccagccac
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A.2

EF526216 441 bp mRNA linear ROD 21-MAY-2007
DEFINITION Marmota monax NKp46 mRNA, partial cds.
ACCESSION EF526216
SOURCE Marmota monax (woodchuck)
AUTHORS Guy, C.S. and Michalak, T.I.
TITLE Direct Submission
FEATURES Location/Qualifiers
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DS
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ORIGIN

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121 cgtagctcg ggagcacaca ggcagagttc cctgtgggcc cggtgactgt gggccaccag
181 gggacctaca gatgctttgg ctctacaac aaccacgat ggtcttccc cagcaatccg
241 gtgatgctc tggtagacag aggtgcgggg aacagcagtc ttgcacccac agacccgacg
301 gattccccg attctgtga cccctacctg gtgaccacag agtcggggtc ccagaaagac
361 cccaccgct gggaccacac agcccagaac ctctccgga tgggcctggc gttctggtg
421 ctggtggtc tggcgtggt c
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A.3

EF621767 672 bp mRNA linear ROD 19-AUG-2007
DEFINITION Marmota monax CD1d mRNA, partial cds.
ACCESSION EF621767
SOURCE Marmota monax (woodchuck)
AUTHORS Guy, C.S. and Michalak, T.I.
TITLE Direct Submission
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ORIGIN

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541 cagcagggca ctcagagagg tgacatcctg cccaatggtg atgagacgtg gtatctccga
601 gcaaccctgg atgtggcagc tggggagctg gctggcctgg cctgcagggt gaagcacagc
661 agcctaggag gg

A.4

EF621766 309 bp mRNA linear ROD 19-AUG-2007

DEFINITION Marmota monax CD8 mRNA, partial cds.

ACCESSION EF621766

SOURCE Marmota monax (woodchuck)

AUTHORS Guy, C.S. and Michalak, T.I.

TITLE Direct Submission

FEATURES Location:Qualifiers

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ORIGIN

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181 ctggacttcg cctgtgacat ctacatctgg gcgcccttgg ctgggacctg cgcggtcct
241 ctgttgacac tggatcacac tgcatctgt caccaccgga aacgaaggcg tgtttgcaaa
301 tgcgcccg

A.5

EF621765 975 bp mRNA linear ROD 19-AUG-2007

DEFINITION Marmota monax CD4 mRNA, partial cds.

ACCESSION EF621765

SOURCE Marmota monax (woodchuck)

AUTHORS Guy, C.S. and Michalak, T.I.

TITLE Direct Submission

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ORIGIN

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A.6

EF216348 220 bp mRNA linear ROD 12-FEB-2007

DEFINITION Marmota monax interleukin 8 mRNA, partial cds.

ACCESSION EF216348

SOURCE Marmota monax (woodchuck)

AUTHORS Pohling, J., Guy, C.S. and Michalak, T.L.

TITLE Direct Submission

FEATURES Location/Qualifiers

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ORIGIN

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121 agaactgaga gtgattgaga gtggaccaca ctgtgccaat tcagaaatca ttgtcaagct
181 tgttgatgga agagagcttt gcctgaacct caaggaaaaa

A.7

AY993961 606 bp mRNA linear ROD 20-APR-2005
 DEFINITION Marmota monax granzyme B mRNA, partial cds.
 ACCESSION AY993961
 SOURCE Marmota monax (woodchuck)
 AUTHORS Guy, C.S. and Michalak, T.I.
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ORIGIN

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421 agtcagaaag caccaggtgt gcgagcgcga ggaattatta aaagagtact acaagagtag
481 catccagata tgtgtggggg atccaaagga aaacaaagct tccttcagg gggactccgg
541 aggccctctt gtgtgtaacc atgtggccca aggaattgtc tttatggaa ataaaaatgg
601 aaacct

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A.8

AY993960 499 bp mRNA linear ROD 20-APR-2005
DEFINITION Marmota monax FAS antigen CD95 mRNA, partial cds.
ACCESSION AY993960
SOURCE Marmota monax (woodchuck)
AUTHORS Guy,C.S. and Michalak,T.I.
TITLE Direct Submission
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QKLQLL"

ORIGIN

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241 tagcttttgg gatatacaga attttaagt accgcagta aaaacatgt caccatgaac
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361 acatcattag tattctgaa gagatgacga tcaatcaagt tagagaatt gtctgtaaga
421 atggtatcaa tgaagccaag atagatgaga tcaagaatga caatcgcaa gatacagctg
481 agcagaaact ccagctgct

